CHARACTERIZATION AND REGULATION OF RENAL VASOPRESSIN RECEPTORS

By

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This dissertation is dedicated to my parents, Lillian and Murry Steiner.

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KEY TO ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ADH	antidiuretic hormone
ANF	atrial natriuretic factor
ANG II	angiotensin II
ANOVA	analysis of variance
ATCC	American Tissue Culture Collection
ATP	adenosine triphosphate
AVP	arginine vasopressin
BSA	bovine serum albumin
cAMP	cyclic adenosine 3′,5′-monophosphate
cGMP	cyclic guanosine 3′,5′-monophosphate
CNS	central nervous system
CPM	counts per minute
DAME	D-ala-met-enkephalinamide
DDAVP	deamino-D-arginine vasopressin
DG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DOCA	desoxycorticosterone acetate
DPM	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
FBS	fetal bovine serum
GTP	guanosine triphosphate

IBMX	isobutylmethylxanthine
IP	inositol phosphate
KIU	kallikrein inhibitory units
KRB	Kreb's Ringer's buffer
LVP	lysine vasopressin
MDCK	Madine-Darby canine kidney
NTS	nucleus of the tractus solitarius
OXY	oxytocin
PBS	phosphate-buffered saline
PI	phosphatidyl inositol
PMSF	phenylmethylsulfonyl fluoride
PNPP	p-nitrophenyl phosphate
PVN	paraventricular nucleus
RIA	radioimmunoassay
RPM	revolutions per minute
SEM	standard error of the mean
SHR	spontaneously hypertensive rat
SON	supraoptic nucleus
TFA	trifluoroacetic acid
WKY	Wistar-Kyoto

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CHARACTERIZATION AND REGULATION OF RENAL VASOPRESSIN RECEPTORS

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Vasopressin receptors were characterized on basolateral membranes of renal tubular epithelia and their capacity for regulation was studied during alterations in fluid balance. Membranes were obtained from rat kidney and prepared by differential and density gradient centrifugation techniques. Radioreceptor methodologies were used to demonstrate that binding was rapid, reversible, specific and saturable. The binding site displayed a pharmacological profile for the V_2 receptor subtype, based on competition studies with selective V_1 and V_2 antagonists.

Vasopressin receptors were impacted by the physiological state of the animal. Water-deprived animals displayed significantly elevated plasma vasopressin levels. As a consequence vasopressin binding sites were downregulated. Conversely, the administration of the

mineralocorticoid desoxycorticosterone acetate (DOCA) to rats resulted in the increased expression of tubular vasopressin receptors and the development of DOCA-salt hypertension. This was associated with an increase in adenylate cyclase activity in the basolateral membrane preparations. These data provide a cellular basis for the increased tubular sensitivity to vasopressin and its fluid-retaining and sodium-reabsorbing properties that are characteristic of this volume-dependent model of hypertension. The heightened expression of vasopressin receptors appears to result from an increased transcriptional rate of protein synthesis induced by the mineralocorticoid DOCA.

Desoxycorticosterone acetate (DOCA) was ineffective in regulating vasopressin receptors or adenylate cyclase activity in LLC-PK_1 cells, a cell line of renal epithelial origin. This coincides with a lack of mineralocorticoid receptors in this particular cell strain. Signal transduction mechanisms were also evaluated and vasopressin stimulated cyclic AMP formation and phosphatidyl inositol hydrolysis, suggesting that these cells possess both V_1 and V_2 receptor subtypes. This demonstrates that one hormone can modulate cellular activity by interacting with multiple effector systems.

These studies demonstrate that renal vasopressin receptors are influenced by the physiological state of the animal. Similarly, receptor-coupled signal transduction mechanisms are subject to modification, in vitro. Alterations in vasopressin receptors or receptor-mediated processes may provide a cellular basis for pathophysiological conditions associated with fluid and electrolyte balance.

CHAPTER 1
BACKGROUND

Arginine^{8}-vasopressin (AVP) is an ancient hormone. It is one of the most phylogenetically conserved, presumably because of its importance for water balance in early invertebrates (Sawyer, 1977). Scientifically, it is an old hormone. Oliver and Schaefer (1895) first described the rise in systemic blood pressure following the infusion of a whole pituitary extract. The renal antidiuretic actions of the hormone were first noted by Starling and Verney (1924). A wealth of information has accumulated on the physiological actions of vasopressin since these pioneering studies. It is now clear that vasopressin not only serves as the principal hormone governing fluid homeostasis, thereby requiring the antidiuretic hormone (ADH) nomenclature, but also plays a role in cardiovascular control. In addition, various central nervous system (CNS) effects have been ascribed to the hormone.

Synthesis, Transport and Release of Vasopressin

Arginine^{8}-vasopressin and oxytocin both are closely related nonapeptides, characterized by cysteine residues linked by disulfide bridges. Often classified as posterior pituitary hormones, the hormones are actually produced within specialized neurons in the hypothalamus. Whereas the biologically active molecules are small molecular weight peptides, they are synthesized, transported and stored in secretory granules in association with specific carrier proteins or neurophysins. Sachs and Takabatake (1964) first

demonstrated that the vasopressin-neurophysin molecule is formed as part of a common precursor hormone. The precursor molecule is synthesized in the neuronal cell body by a ribosomal mechanism and packaged into secretory granules by the Golgi apparatus. The vesicles are transported by axoplasmic flow toward the nerve terminal in the neural lobe of the pituitary where they are stored. Gainer et al. (1977) have shown that the prohormone undergoes post-translational processing during axonal transport, suggesting that the granules must contain both the precursor molecule and enzymes responsible for its modification. The contents of the secretory granules (hormone, neurophysin, and residual peptide fragments) are released upon depolarization of the nerve terminal. This exocytotic process involves a calcium-dependent fusion of the secretory vesicle with the nerve terminal membrane, followed by an opening of the vesicle and an emptying of its contents.

Control of Vasopressin Secretion

It is reasonable to expect that the release of vasopressin from the neural lobe of the pituitary gland should be influenced by factors pertaining to vasopressin's role in fluid homeostasis and circulatory control. Furthermore, regulation of vasopressin secretion would be expected to affect the activity of the paraventricular (PVN) and supraoptic nuclei (SON). Indeed, the activity of these structures is influenced by both osmotic and non-osmotic stimuli.

The pioneering study of Verney (1947) was the first to demonstrate that centrally located structures which were osmo-sensitive, affected the secretion of vasopressin. These structures are so exquisitely sensitive that changes in plasma osmolality as small as

a few milliosmoles, which represents a change of only 1%, can effect the release of vasopressin. Ten years later, experiments conducted by Jewell and Verney (1957) localized the osmoreceptors to the basomedial forebrain. The exact location of Verney's osmoreceptors has been the subject of considerable controversy. While some have claimed that the magnocellular neurons of the PVN and SON are themselves osmosensitive (Leng, 1980), it is now believed that osmosensitive cells within the organum vasculosum of the lamina terminalis may account for the osmotically-induced alterations in vasopressin secretion (Thrasher et al., 1982). This same group has also laid to rest the argument over whether the mechanism for the control of vasopressin release involves osmosensitive neurons or sodium receptors located on the cells within the organum vasculosum laminas terminalis (Thrasher et al., 1980). The neural connections which relay information between cells in this circumventricular region and the PVN and SON have been described by Miselis (1981).

Non-osmotic control of vasopressin release is probably more important for cardiovascular regulation. Thus, hypovolemia, hemorrhage or decreased cardiac output will elicit the secretion of AVP, for the purpose of maintaining circulatory homeostasis. This type of control arises from high pressure baroreceptors in the carotid sinus and aortic arch and low pressure volume receptors in the left atrium and pulmonary veins. The afferent impulses of this neurohumoral arc are carried by the ninth and tenth cranial nerves to their respective nuclei in the brainstem. Multisynaptic pathways then transmit this information to the PVN and SON of the

hypothalamus. The osmotic and non-osmotic control of vasopressin secretion has been reviewed by Schrier et al. (1979).

The synaptic inputs to the PVN and SON of the hypothalamus which are involved in the control of vasopressin secretion are less well understood (Morris, 1983). The nature of the neurotransmitters and neuromodulators involved in these processes has only recently begun to be addressed (Sladek, 1983). Angiotensin II, another peptide hormone involved in blood pressure control and fluid homeostasis, has been shown to stimulate the release of vasopressin, following intracerebroventricular administration (Phillips, 1987). Cholinergic parasympathetic pathways subserve both the cardiovascular- and volume-related functions of AVP release. The ascending noradrenergic pathways from the medullary cardiovascular centers appear to exert a tonic inhibitory affect on vasopressin release. In addition, dopamine and gamma-aminobutyric acid also are inhibitory. The actions of other putative mediators of vasopressin release has recently been reviewed by Sklar and Schrier (1983).

Biological Actions of AVP in the Periphery

Since the original observation of a vasopresssin-induced pressor response by Oliver and Schaefer (1895), numerous other biological effects of vasopressin have subsequently been noted. Many of the observations pertain to the fluid-retaining and cardiovascular properties of the hormone, or are in concert with other systems that subserve these functions.

Renal Effects

It is now clear that vasopressin acts at multiple sites within the kidney to affect renin release, glomerular filtration, NaCl transport

and water reabsorption. Vasopressin is a potent inhibitor of renin secretion. Subpressor doses of AVP decrease plasma renin levels. It is thought that vasopressin acts directly on the juxtaglomerular cells, as the effect can be demonstrated in the isolated kidney and occurs with no demonstrable effect on renal hemodynamics (Vander, 1968).

Arginine^{8}-vasopressin reduces the glomerular ultrafiltration permeability coefficient (K_f) and may decrease the glomerular filtration rate (Ichikawa and Brenner, 1977). While the mechanism by which AVP decreases the K_f is not clear, Ausiello et al. (1980) have shown that vasopressin stimulates the contraction of glomerular mesangial cells grown in culture. Therefore, it is possible that mesangial contraction reduces the surface area available for glomerular ultrafiltration.

The next demonstrable action of vasopressin occurs at the thick ascending Limb of Henle. Morel et al. (1976) made the first observation of vasopressin-sensitive adenylate cyclase activity in both the medullary and cortical portions of the thick ascending limb. The concentration of AVP necessary to stimulate adenylate cyclase activity in the thick ascending limb is one or two orders of magnitude higher than that needed in more distal tubular segments. The physiological significance of this finding remained in doubt until Hebert et al. (1981a) found that AVP, in pico-molar concentrations, stimulated NaCl transport out of the tubular lumen into the interstitium. This suggests a role for vasopressin in the maintenance of the corticomedullary interstitial osmotic gradient.

The major target for the action of vasopressin is the distal segment of the collecting tubule. Here vasopressin, through a cAMP-

dependent mechanism, can exert its well known antidiuretic effect, by altering the permeability of the tubular membrane to water (Grantham and Burg, 1966). The cellular events beyond cAMP formation include stimulation of cAMP-dependent protein kinase in the cytosol and protein kinase A-dependent phosphorylation of the apical membrane, resulting in an increased permeability of the apical membrane to water (Figure 1-1). At present, the precise mechanism by which protein kinase activation, calcium activation and microtubule-microfilament assembly result in an alteration in membrane permeability remain unclear, despite the well-established antidiuretic effect of the hormone. The most favored hypothesis to emerge, however, is that vasopressin induces, by some conformational change, the formation of aqueous channels in the apical membrane (Gluck and Al-Awqati, 1980). This is supported by the appearance of ADH-induced intramembraneous particle aggregates in the luminal membrane. The number of aggregates and the cumulative area of membrane occupied by the particles are directly related to the magnitude of ADH-induced changes in water permeability (Muller et al., 1980).

Cardiovascular Effects

Although an elevation of blood pressure was the first physiological effect noted following the administration of vasopressin, the cardiovascular actions of vasopressin have generally been regarded as physiologically unimportant. This is somewhat surprising as vasopressin has been shown to be one of the most potent vasoconstrictive agents (Altura and Altura, 1984). In addition, even the antidiuretic action of the hormone contributes to the regulation

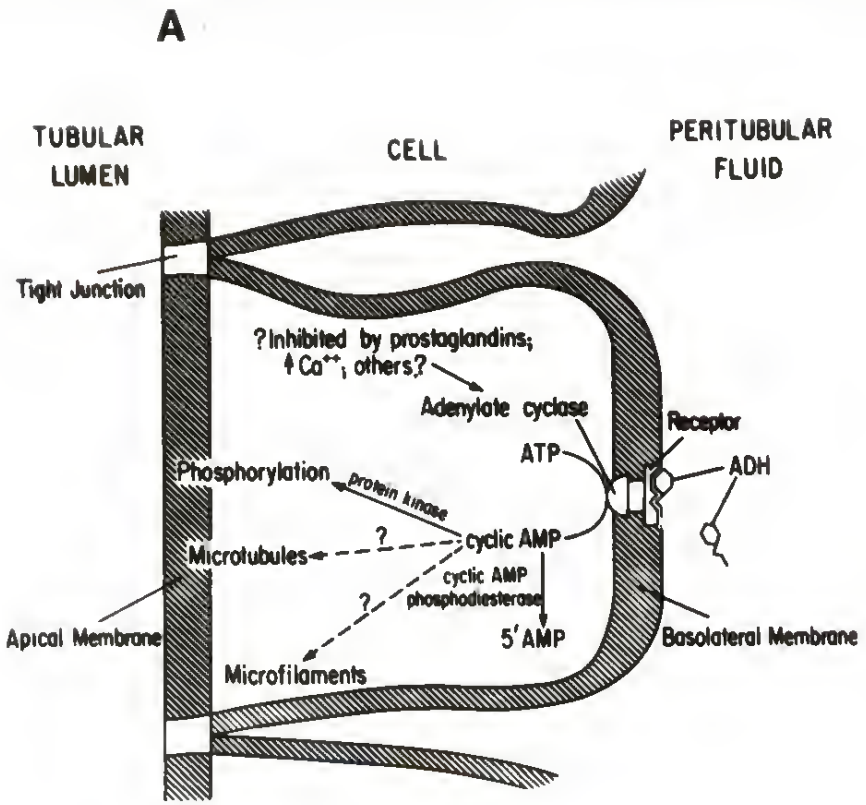

Figure 1-1. Model of antidiuretic hormone (ADH)-stimulated adenylate cyclase cascade in renal tubular epithelial cell. ADH combines with receptor in basolateral membrane. Interaction of hormone and receptor stimulates adenylate cyclase, resulting in the formation of cyclic AMP and the activation of cAMP-dependent protein kinases. These enzymes act to phosphorylate microtubules, microfilaments or integral proteins in the apical membrane. While the mechanism of increased apical permeability to water remains unclear, the most widely accepted model postulates the formation of aqueous channels (Gluck and Al-Awqati, 1980).

of extravascular volume and exerts hemodynamic effects even at subpressor doses (Cowley et al., 1984b). Guyton and others propose that the antidiuretic action of the hormone can even produce hypertension under certain conditions (Manning et al., 1979). The cellular basis for this phenomenon is alluded to in Chapter 4 of this dissertation.

Vasopressin has been shown to exert direct negative chronotropic and inotropic effects in vitro (Nakashima et al., 1982). The physiological significance of these direct cardiac effects remains in question as exceedingly high concentrations of vasopressin were necessary to elicit the observed effects.

Vasopressin is a potent vasoconstrictor, in vivo and in vitro. In vivo, administration of vasopressin leads to an increase in total peripheral resistance from the vasoconstriction of a variety of vascular beds. The skin, muscle and splanchnic circulations are the most sensitive, followed by the mesenteric and coronary beds. The renal, hepatic and cerebral circulations are unaffected by AVP (Liard, 1984). The vasoconstrictive potencies of several pressor substances and a number of vasopressin analogues has recently been evaluated in an isolated smooth muscle preparation and AVP was determined to be the most potent agent tested, although the in vitro activity did not exactly parallel the in vivo activity (Altura and Altura, 1984). However, when vasopressin is infused into conscious intact animals, blood pressure does not normally rise unless exceedingly high plasma levels of vasopressin are attained (>100 pg/ml). Although AVP-induced elevations in total peripheral resistance might occur, arterial pressure is not drastically affected because it is strongly buffered by

the baroreceptor reflex. Furthermore, vasopressin actually tends to enhance the baroreflex by increasing vagal tone and inhibiting sympathetic outflow following i.v. administration, apparently through an interaction with the cardiovascular centers in the brainstem (Cowley et al., 1984a). The role of vasopressin in the central control of the circulation is addressed later in this chapter.

Because the pressor effects of vasopressin are normally obscured by the baroreflex, the physiological importance of AVP in cardiovascular function has often been ignored. Nevertheless, vasopressin plays an important role in the maintenance of blood pressure under conditions of dehydration (Schwartz and Reid, 1983), when circulatory volume is compromised as in the case of hemorrhage (Zerbe et al., 1982), in a situation of adrenal insufficiency (Schwartz et al., 1983) or when the vasoregulatory reflex mechanisms are interrupted. In each of these cases, plasma levels of vasopressin rise in excess of the concentration necessary for maximal antidiuresis. That vasopressin is essential for maintaining circulatory homeostasis in even a moderately compromised condition is evident in the findings of Aisenbrey et al. (1981). In that study, rats were dehydrated for a period of 72 hours and then challenged with a vascular vasopressin blocker. Whereas the blocker had no intrinsic cardiovascular activity nor did it affect blood pressure in normally hydrated animals, blood pressure fell precipitously in the dehydrated rats. In a related study, Brattleboro rats, which are unable to synthesize vasopressin, were unable to maintain their blood pressure when subjected to dehydration (Woods and Johnston, 1983). Collectivelly, these data lend support for a physiological role for vasopressin in circulatory control.

Vasopressin is not a hormone unto itself. It is reasonable to expect that AVP should interact with other regulatory systems to acheive homeostasis. This is most clear in terms of the cardiovascular effects of vasopressin. Vasopressin, the renin-angiotensin system and the autonomic nervous system all interact, to the point of redundancy in maintaining circulatory control. As noted previously, vasopressin can inhibit the secretion of renin from the kidney (Vander, 1968). Conversely, angiotensin has been shown to stimulate the release of AVP (Phillips, 1987). Vasopressin and the autonomic nervous system interact extensively within the brain to acheive circulatory control, as is discussed in a subsequent section. In the periphery, vasopressin potentiates the vasoconstrictor action of norepinephrine and sympathetic nervous stimulation (Bartlestone and Nasmyth, 1965). Conversely, blockade of the sympathetic nervous system greatly accentuates the pressor effect of vasopressin (Pullan et al., 1978). A methodical study by Gavras et al. (1982) provides experimental evidence which supports the interaction of these three effector systems in the maintenance of blood pressure.

Other Biological Effects

While the renal and cardiovascular actions of vasopressin are perhaps the best known, other biological effects have recently been attributed to vasopressin. The central effects of vasopressin in cardiovascular regulation will be discussed in a subsequent section. Acting as a neuropeptide, AVP has been ascribed a role in thermoregulation, nociception, memory and behavior. Another neuroendocrine action of vasopressin is to cause the release of ACTH from the anterior pituitary, either by acting directly or by

potentiating the effect of corticotropin releasing factor (De Wied, 1983). A metabolic action has been attributed to vasopressin, by virtue of its glycogenolytic effect in the liver (Michell et al., 1979). Steroidogenic effects have been attributed to vasopressin in the Leydig cells of the testes and the glomerulosa cells of the adrenals (Nicholson et al., 1984). It has also been shown that vasopressin is involved in platelet aggregation (Haslam and Rosson, 1972).

Vasopressin in the Central Nervous System

Vasopressinergic Projections

Vasopressin (and oxytocin) are synthesized in neurosecretory neurons located within the paraventricular and supraoptic nuclei of the hypothalamus. It was originally thought that oxytocin was exclusively formed in the SON and that vasopressin was produced only in the PVN. It is now clear that both peptides are synthesized in separate and topographically distinct populations of neurons within each nucleus (Swanson and Sawchenko, 1983). Each nucleus is characterized cytoarchitecturally by the presence of large magnocellular neurons and small, round parvocellular neurons. It has been known since the work of Cajal (1894) that the magnocellular neurons of the SON project to the neural lobe of the pituitary. While morphologically quite similar, it was not recognized until forty-two years later that the magnocellular neurons of the PVN also project to the posterior pituitary (Ingram et al., 1936). Although the findings of Cajal remain unchanged, histological evidence concerning vasopressinergic projections from the PVN has been greatly expanded. Magnocellular projections from the PVN have been traced to the external lamina of the median eminence (Diedrickx et al., 1976).

Maraka et al. (1981) have suggested that vasopressin released into this capillary portal zone may affect the synthesis and release of ACTH and other adenohypophyseal hormones.

Parvocellular neurons of the PVN, which are morphologically and topographically distinct from magnocellular neurons, have recently been shown to project to neural lobe of the pituitary (Swanson and Kuypers, 1980). Vasopressinergic fibers from the hypothalamus are reported to innervate the lateral septum in the forebrain (Sofroniew and Weindl, 1978). More extensive descending projections have been traced to the intermediolateral column of the spinal cord, the nucleus tractus solitarius and the dorsal vagal complex in the brainstem (Buijs, 1978; Swanson and Kuypers, 1980) and the locus coeruleus (Sofroniew, 1980). Despite the complex organization of the PVN, Swanson et al. (1980), using retrograde labeling techniques, have convincingly demonstrated that separate neurons project to each of the areas described above.

Afferent Projections to the PVN

The development of histofluorescence methods lead to the demonstration that the PVN and the SON contained one of the highest densities of noradrenergic terminals in all of the brain (Carlsson et al., 1962). By a variety of anterograde and retrograde labeling techniques, the origins of these synaptic inputs have been traced to A_1 and C_1 cell groups of the ventral medulla, the A_2 and C_2 cell groups in the medial NTS and the A_6 cell group in the locus coeruleus. The parvocellular neurons of the PVN are preferentially innervated by noradrenergic axons from the medulla and locus coeruleus (A_2 and A_6) while the A_1 area of the ventrolateral medulla

projects to magnocellular and parvocellular neurons of the PVN and SON. In addition to rostral projections from the brainstem, the PVN receives numerous inputs from diencephalic and telencephalic structures. The efferent connections of the PVN and the complex organization of this hypothalamic nucleus have been elegantly reviewed by Swanson and Sawchenko (1983).

The ascending catecholaminergic pathways and descending vasopressinergic pathways provide an anatomical basis for the feedback loops which enable vasopressin to influence autonomic and cardiovascular function and in turn, allow for adrenergic control of vasopressin release. This latter point may account for the tonic inhibitory influence that α-adrenergic stimuli have on vasopressin secretion (Kimura et al., 1981).

Role of AVP in Central Cardiovascular Regulation

The diversity of afferent and efferent projections of the PVN has lead Swanson and Sawchenko (1980) to view this vasopressin-synthesizing site as a visceral effector nucleus, capable of influencing the secretion of hormones from the anterior and posterior lobes of the pituitary and neural activity in the sympathetic and parasympathetic divisions of the autonomic nervous system. The vasopressinergic innervation of certain brainstem and forebrain nuclei tends to suggest that AVP plays an important role in the central control of circulation, exclusive of its well known vasoconstrictive action in the periphery.

One potential role of AVP in the central control of cardiovascular regulation involves its ability to modulate neuronal activity of the NTS, which constitutes the anatomical substrate of the

baroreceptor reflex. The NTS receives information from afferent baroreceptor fibers originating in the carotid sinus and aortic arch. The NTS influences cardiovascular function in three ways: (a) it relays baroreceptor activity to other brainstem areas to modulate spinal preganglionic neuronal activity; (b) it relays baroreceptor and chemoreceptor activity to the hypothalamus, which serves to regulate and integrate autonomic and cardiovascular responses; and (c) it participates in the regulation of fluid and electrolyte homeostasis by influencing the release of AVP (Reis et al., 1984). Experimental evidence tends to support the hypothesis that bloodborne AVP enhances the sensitivity of the baroreceptor efferent pathway. Courtice et al. (1984) have electrophysiological data which show that AVP enhances the baroreflex by increasing parasympathetic vagal tone and depressing barosympathetic outflow. In addition, Brattleboro rats, which are deficient in vasopressin, have a depressed baroreflex, which can be corrected by intravenous infusions of vasopressin (Imai et al., 1983). Though somewhat paradoxical, centrally administered AVP acts in an opposite manner to peripherally administered AVP. Intracerebroventricular administration of AVP elcits a pressor response, tachycardia, increased sympathetic outflow and increased levels of plasma catecholamines (Unger et al., 1984). Matsuguchi et al. (1982) reported that the microinjection of AVP directly into the NTS caused increases in arterial pressure and heart rate. Berecek et al. (1984) reported similar findings following microinjections of AVP into the locus coeruleus.

In addition to the reciprocal neuroanatomical pathways between the PVN and NTS that are described above, functional interactions

have also been noted. Lesions of the PVN prevent the elevation in arterial pressure and heart rate associated with aortic baroreceptor deafferentation (Zhang et al., 1983). Employing electrophysiological techniques, Ciriello and Calaresu (1980) have shown that the NTS and the dorsal motor nucleus of the vagus relay cardiovascular information directly to the PVN. In the same study it was also shown that electrical stimulation of the PVN lead to an increase in arterial pressure and heart rate and inhibited the reflex bradycardia evoked by stimulation of the carotid sinus nerve. Conversely, destruction of the A_1 region, the group of noradrenergic cell bodies thought to tonically inhibit AVP secretion, results in a condition of fulminating hypertension, marked by high levels of vasopressin in the plasma (Blessing et al., 1982).

Thus, a feedback loop appears to exist between the PVN and the NTS which serves to modulate the ability of the baroreflex to regulate arterial pressure. Since the NTS is the primary afferent relay for baroreceptor fibers and the dorsal motor nucleus of the vagus contains vagal cardioinhibitory preganglionic neurons, projections to these structures from the PVN appear to participate in the central control of cardiovascular regulation. Vasopressin also appears to influence other sites in the central nervous system involved in circulatory control, such as the locus coeruleus. A vasodepressor function has been attributed to the action of vasopressin in the dentate gyrus of the hippocampus (Versteeg et al., 1984).

Vasopressin Receptors

Current dogma holds that all peptide or protein hormones initiate their actions by binding to membrane-bound receptors located on the cell surface of the target tissues. The interaction of the hormone with its receptor causes the activation of one of a number of second messenger systems (cAMP, cGMP, PI). Depending on the tissue and receptor involved, this can result in the activation of enzymes, mobilization of intracellular stores of calcium (which, in turn can affect enzyme activity), phosphorylation of membrane proteins or the activation of ion channels, leading to the appropriate biological response.

AVP interacts with at least two subclasses of vasopressin receptors, depending on the tissue in question. Michell et al. (1979) formally designated these different subtypes as V_1 and V_2 vasopressin receptors on the basis of the effector systems to which these receptors are coupled. Elucidation of the subtypes has been greatly facilitated by the synthesis of structurally distinct vasopressin analogues which bind differentially to the V_1 or V_2 receptor. Development of these analogues has resulted in the design of specific agonists and antagonists for each of the receptor subtypes (Sawyer et al., 1981).

V_1 receptors are coupled to phospholipase C and the hydrolysis of phosphatidyl inositol (PI) with the subsequent formation of inositol phosphates (IP) and diacylglycerol (DG). Inositol phosphates have been shown to increase the mobilization of intracellular stores of calcium. Diacylglycerol on the other hand, activates the enzyme protein kinase C. V_2 receptors are coupled to adenylate cyclase and

the formation of cAMP. Both V_1 and V_2 receptors are functionally linked to their respective effector systems through guanine nucleotide regulatory proteins (G proteins). Thus, the antidiuretic action of vasopressin is mediated through V_2-type receptors while the vasoconstrictive, glycogenolytic and steroidogenic actions of AVP are mediated by V_1-type receptors, based on structure-activity relationship studies with vasopressin analogues or by directly determining the second messenger. A novel vasopressin receptor (V_{1b}) which displays a pharmacological profile that does not clearly fall into one category or the other has been reported to mediate the effects of AVP in the anterior pituitary (Antoni, 1984). Furthermore, recent evidence suggests that at least one tissue may possess both V_1 and V_2 receptor subtypes (Chapter 6).

Kidney

Evidence for the binding of vasopressin to hormone-responsive cells in the kidney was first suggested by the autoradiographic studies of Darmady et al. (1960). Specific binding of ^{3}H-AVP to plasma membranes of the renal medulla was subsequently reported by two independent laboratories (Campbell, et al., 1972; Bockaert et al., 1973). Neither study succeeded in localizing the binding to plasma membranes of renal tubular origin nor were they able to distinguish between basolateral and apical membranes. However, Bockaert et al. (1973) and others (Nakahara et al., 1978) were able to demonstrate the correlation between receptor occupancy and the activation of adenylate cyclase. These studies were consistent with the finding that cAMP and vasopressin-induced changes in membrane permeability were only noted following application to the peritubular side of

isolated renal tubules (Grantham and Burg, 1966). It may be concluded that these vasopressin binding sites constitute the receptors which mediate the antidiuretic action of the hormone in the distal tubular collecting segments of the nephron. Since then, vasopressin binding sites have been characterized on a variety of membranes prepared from porcine, bovine, rat and human kidneys (Jard, 1983). A more convincing arguement for the epithelial localization of vasopressin binding sites comes from the finding of Roy and Ausiello (1981a). Using tissue culture techniques, these investigators were the first to characterize vasopressin receptors in LLC-PK_1 cells. This cell line is derived from tubular elements of the pig kidney (Hull et al., 1976). Both intact cells and renal membranes all appear to possess a single class of high affinity binding sites (K_D = 1-25 nM).

All vasopressin-sensitive renal tissue need not be of epithelial origin, however. Vasopressin receptors have been characterized on glomerular mesangial cells (Jard et al., 1987) and appear to mediate the vasopressin-induced contraction of this tissue (Ausiello et al., 1980). This action may account for the alteration in glomerular ultrafiltration observed with vasopressin in the intact glomerulus. Vasopressin binding sites in intact glomeruli has recently been reported (Stoeckel et al., 1987). Thus, caution must be employed when interpreting vasopressin binding studies conducted in the kidney by autoradiography or when using crude membrane preparations.

Vasculature

In order to effect the cardiovascular actions of vasopressin mediated through its vasoconstrictive properties, it follows that

vasopressin binding sites must be present on the smooth muscle cells of the resistance vasculature. This observation has proven difficult due to the high degree of cellular heterogeneity of the vascular wall and technical problems encountered in preparing pure plasma membrane fractions derived from smooth muscle cells. These obstacles have apparently been overcome as judged by the report by Schiffrin and Genest (1983) describing vasopressin binding in a mesenteric artery membrane preparation. AVP binding has also been demonstrated in aortic smooth muscle cells in primary culture (Penit et al., 1983) and in an established smooth muscle cell line (A-10 cells) (Stassen et al., 1987). These receptors, like those in renal tissue, exhibit nanomolar affinity constants and a single class of binding sites. Whereas renal tubular vasopressin receptors are coupled to adenylate cyclase activation, those in the vasculature stimulate the hydrolysis of phosphatidyl inositol and the mobilization of intracellular stores of calcium, as a means of signal transduction (Aiyar et al., 1986).

Central Nervous System

Vasopressin receptors in the brain have been characterized using traditional biochemical techniques and their distribution analyzed by means of autoradiography (Dorsa et al., 1983). This group found that the receptor behaves kinetically as those found in the periphery. Furthermore, they reported that the NTS in the brainstem displayed a high level of binding. Other investigators have confirmed this finding and extended their observations to include AVP binding in the hippocampus and lateral septum (Biegon et al., 1984). These findings are consistent with the functional, behavioral and immunocytochemical

data which contend that vasopressin acts in the brain as a neurotransmitter or neuromodulator. The presence of AVP receptors in the regions mentioned above parallel the vasopressinergic pathways involved in central cardiovascular regulation.

The nature of the effector system coupled to the vasopressin receptor in the brain still remains in question. Competition of ^{3}H-vasopressin binding with vasopressin analogues suggests that the receptor resembles the vascular or V_1 subtype (Cornett and Dorsa, 1985; Audigier and Barberis, 1985), although no direct measurements of vasopressin-stimulated PI hydrolysis have been reported. However, a presynaptic membrane phosphoprotein known as B50 which is a substrate for protein kinase C, is phosphorylated upon the addition of AVP to rat hippocampal synaptic membranes (Hinko and Perlmutter, 1987). These data tend to suggest that vasopressin acts through V_1-type receptors in neuronal membranes to cause the formation of inositol phosphates and diacylglycerol. DG may activate protein kinase C which in turn, phosphorylates integral membrane proteins. Given the importance of calcium in the process of synaptic transmission, the actions of IP cannot be excluded either.

Other Tissues

A burgeoning number of studies have recently reported the presence of vasopressin receptors in a variety of tissues. ^{3}H-vasopressin binding and its relation to glycogen phosphorylase activity was reported by Cantau et al. (1980). Activation of this enzyme and its resulting glycogenolytic action is mediated by V_1 receptors coupled to PI hydrolysis (Michell et al., 1979). In the anterior pituitary gland, vasopressin receptors were first characterized by

Lutz-Bucher and Koch (1983). Antoni (1984) reported that the ligand specificity of pituitary vasopressin receptors was distinct from previously characterized V_1 and V_2 receptors although no measurements of the second messengers were conducted. Gaillard et al. (1984) however, did report that vasopressin failed to influence adenylate cyclase activity in pituitary membrane fractions, but did potentiate CRF-stimulated cAMP accumulation. Balla et al. (1985) described the binding and signal transduction mechanisms for vasopressin in membranes prepared from rat adrenal capsules (chiefly zona glomerulosa). Displacement studies suggested that the receptor was of the V_1 variety and incorporation studies indicated that AVP promoted ^{32}P uptake into PI. This effect was blocked by V_1 antagonists as was AVP-stimulated PI hydrolysis. The net effect of vasopressin binding in isolated zona glomerulosa cells was the stimulation of aldosterone secretion. Whereas vasopressin stimulates steroid biosynthesis in the adrenals, it inhibits testicular Leydig cell steroidogenesis. Receptors which mediate this effect were described by Meidan and Hsueh (1985). Relative binding potencies of selective agonists and antagonists suggest that it is a V_1 subtype. Finally, a number of cellular elements in the bloodstream are reported to possess vasopressin receptors. Binding sites on platelets were described by Berrettini et al. (1982). Mononuclear phagocytes were shown by Block et al. (1981) to possess vasopressin receptors. Although AVP has been shown to cause platelet aggregation, the function of vasopressin receptors in monocytes is unknown. Table 1-1 summarizes the current state of knowledge concerning vasopressin receptors in biological tissues and their significance.

TABLE 1-1.
BIOLOGICAL ACTIONS OF ARGININE^8-VASOPRESSIN.

Tissue	Action	Receptor Subtype	Reference
Brain	Neuromodulator: cardiovascular control, nociception, thermoregulation	V_1	DeWied, 1983
Anterior pituitary	ACTH release	V_{1b}	Antoni, 1984
Platelets	Aggregation	?	Berrettini et al., 1982
Mononuclear phagocytes	?	?	Block et al., 1981
Blood vessels	Vasoconstriction	V_1	Schiffrin & Genest, 1983
Liver	Glycogenolysis	V_1	Michell et al., 1979
Adrenal glomerulosa cells	Steroidogenesis	V_1	Balla et al., 1985
Testes	Inhibits androgen biosynthesis	V_1	Meidan & Hsueh, 1985
Juxtaglo-merular cells	Inhibits renin secretion	?	Vander, 1968
Glomerular mesangial cells	Contraction	V_1	Jard et al., 1987
Renal tubular epithelium	Solute, ion transport	V_2	Morel et al., 1987

Significance

Myriad biological functions have now been attributed to vasopressin. The preponderance of evidence suggests that its major action pertains to its role in circulatory and fluid homeostasis. This is achieved in a concerted manner, through fluid and electrolyte reabsorption, vasoconstriction, modulation of the baroreflex and central regulation of cardiovascular centers.

The actions of vasopressin can be controlled in a number of ways. One is to control vasopressin secretion itself. The levels of circulating AVP are regulated by osmotic and non-osmotic mechanisms. Endocrine feedback systems also act to control AVP release. For example, dehydration will cause an osmotically-mediated release of vasopressin but, in addition, will activate the renin-angiotensin system in the brain, which in turn will stimulate the release of AVP.

The action of vasopressin can also be affected at the target tissue. This usually implies some type of modulation of hormone receptor or post-receptor modification. The concept of hormone receptor regulation has been the subject of several recent reviews (Catt et al., 1979; Roth and Taylor, 1982; Lefkowitz et al., 1984). Receptor regulation can occur by "homologous" or "heterologous" mechanisms. The former implies that a hormone can modulate the cell membrane content of its own specific receptor. Alternatively, receptor number may remain the same but the system is desensitized through an uncoupling of the receptor from the signal transducing elements, often by covalent modification of the receptor. Heterologous regulation suggests that other hormones or factors can

regulate the receptor of a second hormone. For example, a steroid hormone may affect the expression of a peptide hormone receptor through transcriptional events. In any case, regulation of receptors can affect the cellular sensitivity to hormonal stimulation. For the antidiuretic hormone, this is of tanamount importance in its ability to maintain fluid homeostasis.

The studies delineated in this dissertation were conducted to (a) characterize antidiuretic hormone receptors in the kidney, (b) study their physiological regulation in vivo, by altering the state of fluid balance, ranging from a condition of dehydration to a volume-expanded model of hypertension, and to (c) establish a "parallel" system in vitro, to examine receptor regulation and signal transduction mechanisms at the cellular level. Hopefully, this information will expand the current state of thinking about the mechanisms of action of arginine vasopressin and its role in fluid and electrolyte homeostasis and circulatory control.

CHAPTER 2
CHARACTERIZATION OF ^{3}H-VASOPRESSIN BINDING SITES IN RENAL TUBULAR BASOLATERAL MEMBRANES

Introduction

Arginine^8-vasopressin in most species is the mammalian anti-diuretic hormone. It acts upon distal tubular segments of the nephron to promote fluid reabsorption by altering the permeability of the tubular luminal membrane to water. Vasopressin-stimulated activation of the enzyme adenylate cyclase initiates the biochemical cascade responsible for generating transepithelial net fluxes of ions and solutes.

The presence of vasopressin binding sites in the kidney was described as early as 1960 (Darmady et al.) and autoradiographic reports of vasopressin receptors continue up to the present day (Stoeckel et al., 1987). Early studies which utilized traditional biochemical techniques to characterize vasopressin receptors in plasma membranes prepared from renal medullary tissue did so in somewhat crude preparations which may have been contaminated with vascular and non-epithelial elements (Campbell et al., 1972; Bockaert et al., 1973). These types of studies, therefore, fail to distinguish between vasopressin-sensitive cells of tubular epithelial origin from cells which respond to AVP located in the glomerulus and the renal vasculature. Nor do these studies allow one to localize the binding specifically to the basolateral as opposed to the apical region of hormone-sensitive epithelia. Although it is well-established that the vasopressin-

sensitive adenylate cyclase system is present on the peritubular side of tubular epithelia (Grantham and Burg, 1966), the characterization of vasopressin receptors localized specifically to the basolateral membrane has not been previously described. This study was conducted to determine the characteristics of vasopressin binding for a basolateral membrane preparation of rat renal tubular epithelia.

Materials and Methods

Animals and Chemicals

Male Sprague-Dawley rats weighing 200-250g were obtained from the Animal Resource Facility at the University of Florida. Rats were housed individually in 20x18x24 cm wire mesh cages, exposed to a 12:12 hour light:dark cycle, provided a standard Purina rat chow diet and allowed free access to tap water.

Percoll and synthetic peptides were purchased from Sigma Chemical Co. (St. Louis). $d(Et)_2Tyr(Et)D$-AVP (V_1) and $d(CH_2)_5D$-Ile^2-Ile^4-AVP (V_2) vasopressin analogues were generous gifts of Dr. M. Manning of the Medical College of Ohio in Toledo. Tritiated 8-L-arginine [phenylalanine-3,4,5-^{3}H]-AVP (^{3}H-AVP) used in receptor binding studies was purchased from New England Nuclear Corp. (Boston) with a specific activity of 70 Ci/mmol. Other reagents were obtained from Sigma, unless otherwise indicated.

Membrane Preparation

Animals were sacrificed by decapitation. Renal basolateral membranes were prepared according to the density gradient centrifugation method of Boumendil-Podevin and Podevin (1983), with slight modification. Kidneys were removed, decapsulated and rinsed in a chilled buffer containing 250 mM sucrose, 2 mM Tris-HEPES, 0.1

mM PMSF, pH 7.4. The tissue was minced and then homogenized with 5 strokes of a Dounce homogenizer followed by 5 strokes of a teflon pestle homogenizer at a setting of 800 rpm. The resulting homogenate was centrifuged at 1,000 x g for 10 minutes at 4°C. The pellet (P_1) is discarded and the supernatant (S_1) centrifuged at 22,000 x g for 15 minutes at 4°C. The supernatant (S_2) is discarded, leaving a triple-layered pellet (P_2). The upper two layers of the pellet were removed by gentle vortexing and homogenized in 10 ml of the Tris-HEPES-sucrose buffer without PMSF. Percoll (1.2 ml) is added and mixed, then centrifuged at 40,000 x g for 35 minutes at 4°C. The F_1 band (Basolateral membranes, density = 1.037 g/ml) is aspirated and diluted in 5 volumes of 2 mM Tris-HEPES, 85 mM KCl, 85 mM sucrose, pH 7.4. Percoll was removed from the mixture by ultracentrifugation at 60,000 x g for 15 minutes at 4°C. The supernatant is decanted, the membranes are gently washed away from the Percoll pellet, resuspended in Tris-HEPES-sucrose buffer with PMSF and stored in liquid N_2. An outline of this membrane preparation is depicted in Figure 2-1. Protein content was determined with the Folin reagent, using bovine serum albumin as the standard, according to the method of Lowry et al. (1951).

Determination of Na^+K^+-ATPase Activity

The Na^+K^+-ATPase activity was determined by measuring the K^+-stimulated, ouabain-sensitive p-nitrophenylphosphate (PNPP) hydrolysis at pH 7.8, based on the method of Yoshida et al. (1969). Briefly, the assay buffer was composed of 50 mM Tris-HCl (pH 7.8), containing 5 mM $MgCl_2$, 2.5 mM EDTA, 50 mM KCl and 5 mM PNPP. The reaction was carried out in the presence and absence of ouabain (1 mg/ml) by

Kidney tissue homogenized in 2 mM Tris-HEPES, 250 mM Sucrose buffer, pH 7.4 containing 0.1 mM PMSF.

Homogenate → 1K x g, 10'

↓

S_1 → 22K x g, 15'

↓

P_2 (upper two layers of "triple" pellet)

↓

10% Percoll Gradient → 40K x g, 35'

↓

F_1 = Basolateral Membrane (1.037g/ml)

F_2 = Brush Border Membrane (1.041g/ml)

F_3 = Mitochondrial Membrane (1.067g/ml)

Figure 2-1. Scheme for preparation of basolateral membranes from renal tubular epithelia. Kidneys from male Sprague Dawley rats homogenized in Tris-Sucrose buffer containing the protease inhibitor, PMSF. Membranes prepared by density gradient and differential centrifugation, as described in "Methods" section.

the addition of 10 μl membranes (100 μg protein) to 0.5 ml of the assay buffer. The mixtures were incubated for 2-10 minutes at 37°C. The reaction was stopped by the addition of 3.5 ml 1N NaOH and absorbance was read at 410 nm. Net Na^+K^+-ATPase activity was determined by subtracting the PNPP hydrolysis activity in the presence of ouabain from the activity in the absence of ouabain. Specific activity was calculated based on a molar extinction coefficient ϵ = 15,400 $M^{-1}cm^{-1}$, and final values were expressed as units/mg protein (where 1 unit = 1 μmol PNPP hydrolyzed per minute).

Vasopressin Receptor Assay

Membranes, corresponding to 100-200 μg protein were suspended in 300 μl of 100 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 1 mM EGTA and 0.1% BSA, pH 7.3 at 20°C. The incubation buffer contained the peptidase inhibitors aprotinin (1000 K.I.U./ml), leupeptin (1 μg/ml) and pepstatin A (1 μg/ml), to prevent proteolytic degradation of the peptides or radioligand. For saturation studies, triplicate samples were incubated for 45 minutes at 20°C with concentrations of ^{3}H-AVP ranging from 50 pM to 5,000 pM. Non-specific binding was determined in the presence of 5 μM unlabelled AVP. Competitive inhibition by various peptides was assessed in the presence of 1.0 nM ^{3}H-AVP. The incubation was terminated by the addition of 1 ml ice-cold Tris-buffer and the bound tracer was rapidly separated from the unbound moiety by centrifugation at 10,000 x g for 2.5 minutes at 4°C. The membrane pellet was dissolved in 100 μl of formic acid and transferred to a minivial containing 4 ml of liquid scintillation fluid (Liquiscint; National Diagnostics).

Radioactivity was determined in an LKB LS counter. Samples were corrected for variations in efficiency of counting by an internal program and expressed as disintegrations per minute (dpm). Based on the radioactivity and protein in each sample, values are reported as fmoles bound/mg protein. Specific binding was calculated as the difference between total binding and non-specific binding.

Analysis

Data from saturation and competition experiments were analyzed using iterative curve fitting programs. Data are reported as the mean $\pm$ S.E.

Results

The activity of Na^+K^+-ATPase, a marker enzyme for the basolateral membrane was markedly enriched in the F_1 fraction, which displayed a 3.25-fold increase in activity over that of the P_2 pellet and a 10-fold increase when compared to the homogenate (Table 2-1). A two-fold enrichment of AVP binding per unit protein was achieved in the BL membrane preparation compared to the P_2 pellet. A tritiated ligand was chosen for these experiments since iodine destroys the biological activity of the vasopressin molecule (Dorsa et al., 1983). The binding of ^{3}H-AVP to renal basolateral membranes was linearly related to the amount of protein (i.e., membrane) present in the assay (Figure 2-2). Association and dissociation of ^{3}H-AVP is illustrated in Figure 2-3. Equilibrium binding of the ligand occurred within 30 minutes and was stable for up to 2 hours. Upon equilibrium, binding was shown to be reversible by the addition of 1 μM AVP. Specific binding, determined in the presence of an excess amount of AVP was 80-85% of total binding.

TABLE 2-1.
K^+-STIMULATED, OUABAIN-SENSITIVE Na^+-K^+ ATPase ACTIVITY

Fraction	Units*/Mg Protein (x 10^{-3})	Purification
H	0.08 ± .04	1X
P_2	0.26 ± .01	3.25X
F_1	0.80 ± .01	10X

Potassium-stimulated, ouabain-sensitive Na^+-K^+-ATPase activity. Activity determined by measuring K^+-stimulated, ouabain-sensitive p-nitrophenylphosphate (PNPP) hydrolysis at pH 7.8. Values are expressed as units/mg protein, where 1 unit = 1 μmole PNPP hydrolyzed per minute. H, homogenate. P_2, 224,000g pellet. F_1, basolateral membrane fraction. *(1 unit = 10^{-6} moles/min.)

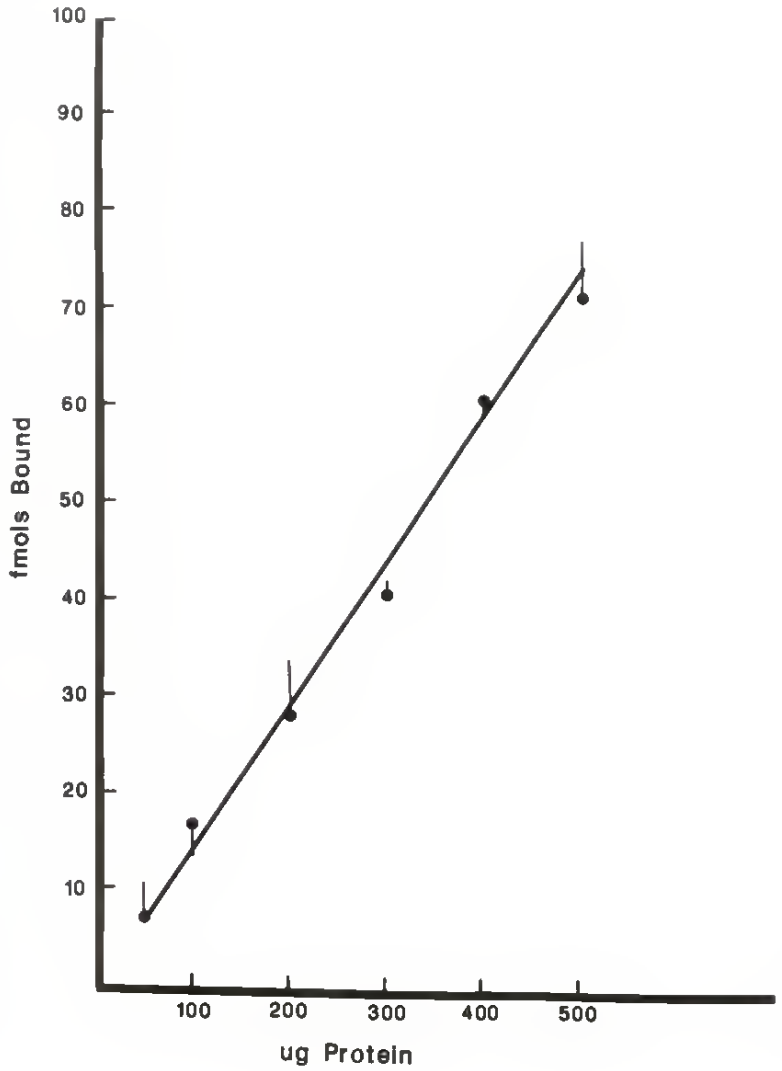

Figure 2-2. ^{3}H-vasopressin binding as a function of basolateral membrane protein concentration. Receptor binding protocol described in "Methods" section. Protein concentration determined by the method of Lowry et al. (1951).

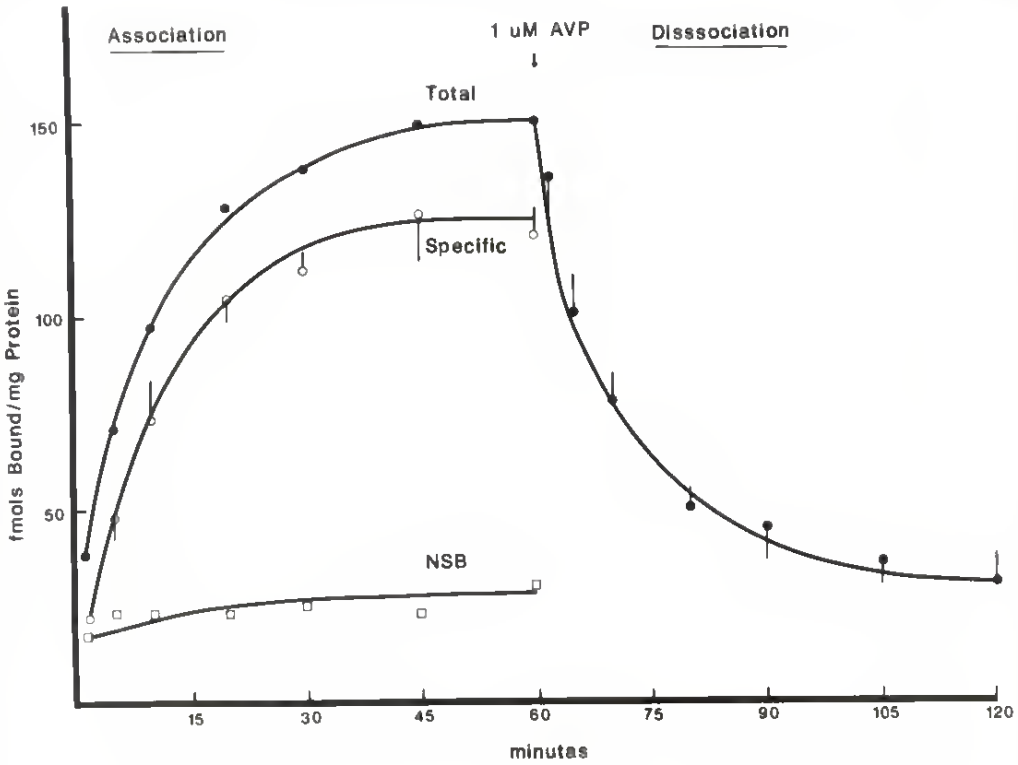

Figure 2-3. ^{3}H-vasopressin (^{3}H-AVP) binding in renal tubular basolateral membranes as a function of time. Receptor binding protocol described in "Methods" section. Association: Total binding determined at 1 nM [^{3}H-AVP]. Non-specific binding (NSB) determined in the presence of 1 nM [^{3}H-AVP] + 1 μM AVP. Total - NSB = Specific, which represents 80-85%. Dissociation: At equilibrium binding (1 hr), 1 nM [^{3}H-AVP] is dissociated from receptor site by the addition of 1 μM unlabeled AVP.

Specificity of the receptor for related AVP analogues and non-related peptides is illustrated in Figure 2-4. The data show that lysine vasopressin (LVP), a vasopressin peptide differing from AVP by only one amino acid in the number 8 position, is some 13 times less effective in displacing the ligand than AVP (IC_{50}: AVP = 0.74 nM vs. LVP = 9.7 nM). Furthermore, the synthetic vasopressin analogue $d(CH_2)_5D\text{-}Ile^2\text{-}Ile^4\text{-}AVP$, a specific tubular ($V_2$) antagonist is some 3,700 times more effective in displacing the ligand than is the vascular (V_1) antagonist $d(Et)_2Tyr(Et)D\text{-}AVP$ (IC_{50}: V_2 = 0.0039 nM vs. V_1 = 14.7 nM). At concentrations of I μM, oxytocin, angiotensin II and D-ala-met-enkephalinamide were incapable of displacing even 50% of the ligand. A representative saturation isotherm for ^{3}H-AVP binding to membranes is depicted in Figure 2-5. Transformation of the data for Scatchard analysis is also represented in Figure 2-5 (insert). Analysis of the data indicates that the maximal binding capacity (B_{max}) for vasopressin receptors on the membranes is ~220 fmol/mg protein and the affinity constant for the receptors is 0.61 nM.

Discussion

Several studies have demonstrated the presence of vasopressin receptors in the kidney (Campbell et al., 1972; Bockaert et al., 1973; Dorsa et al., 1983; Stoeckel et al., 1987). The present study reports the preparation of Na^+K^+-ATPase-enriched renal tubular epithelial basolateral membranes and the characterization of ^{3}H-AVP binding in these membranes. This preparation allows one to separate, by differential and density gradient centrifugation, tubular epithelial membranes possessing vasopressin receptors linked to adenylate

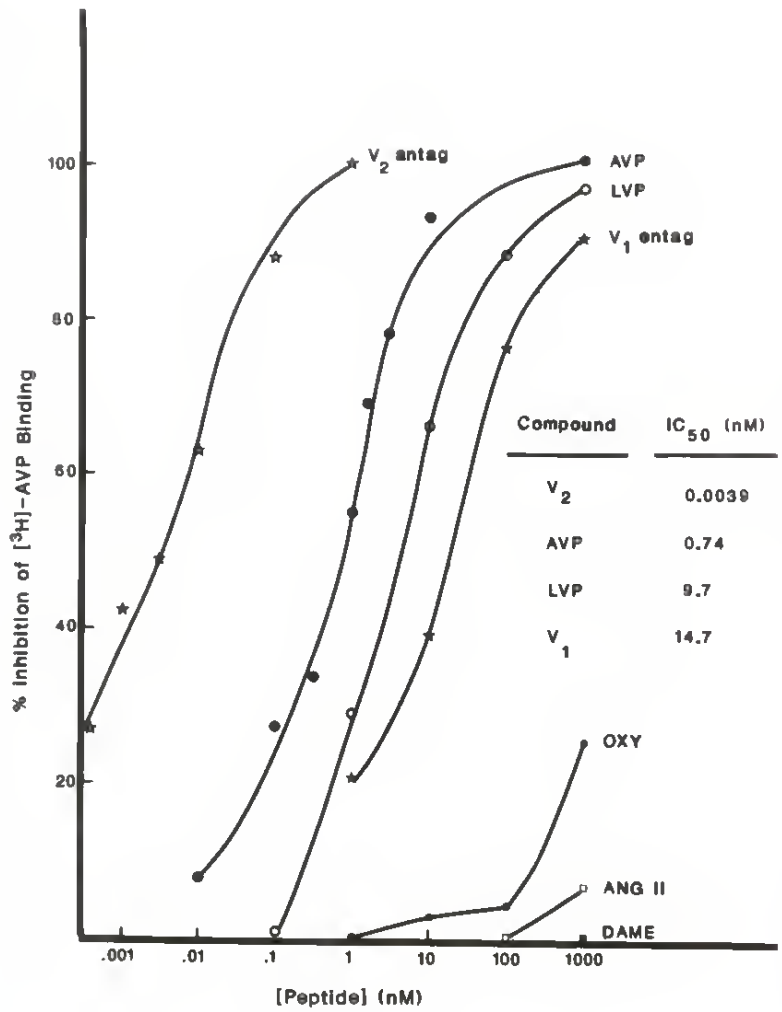

Figure 2-4. Specificity of the vasopressin receptor in renal tubular basolateral membranes. Competition of ^{3}H-vasopressin (^{3}H-AVP) binding at 1 nM by vasopressin analogues and unrelated peptides. Receptor binding protocol described in "Methods" section. V_1 antagonist, $d(Et)_2Tyr(Et)D$-AVP; V_2 antagonist, $d(CH_2)_5D$-Ile^2-Ile^4-AVP; AVP, arginine vasopressin; LVP, lysine vasopressin; OXY, oxytocin; Ang II, angiotensin II; DAME, D-ala-met-enkephalinamide.

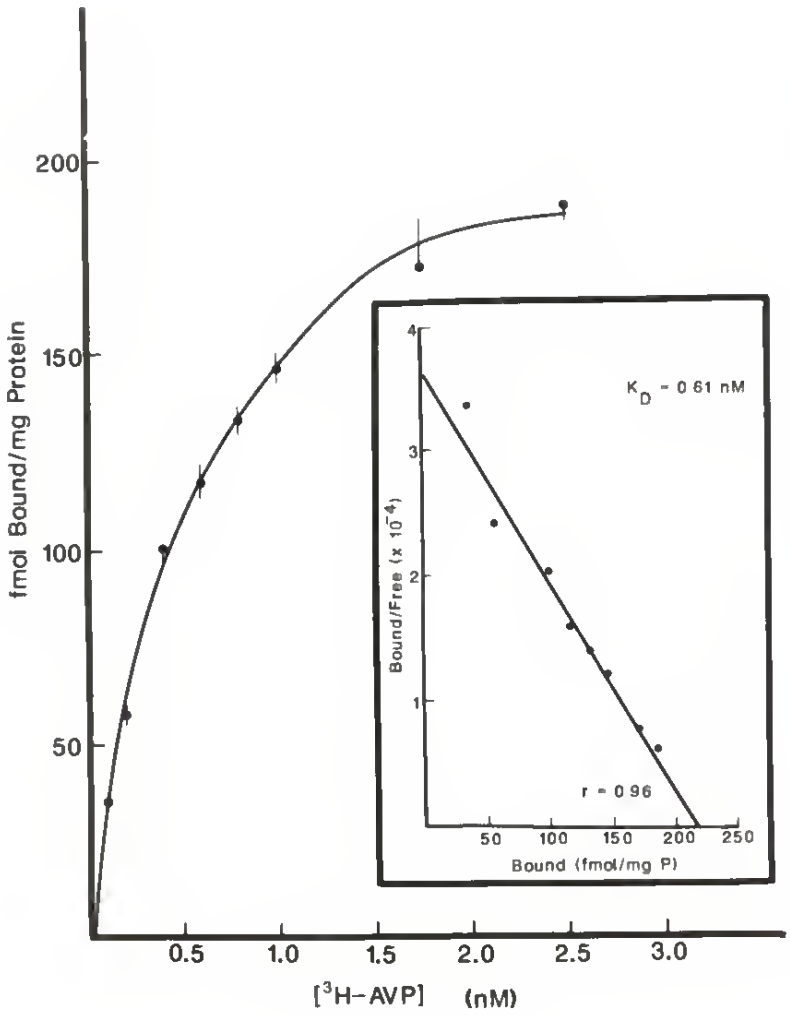

Figure 2-5. Saturation isotherm and Scatchard plot (insert) of ^{3}H-vasopressin (^{3}H-AVP) binding in renal tubular basolateral membranes. Concentration of ^{3}H-AVP ranged from 50 pM to 3,000 pM. Specific binding determined in the presence of 3 μM unlabeled AVP. Details described in "Methods" section.

cyclase (V_2) from vascular endothelial and glomerular mesangial cells possessing vasopressin receptors coupled to the hydrolysis of phosphatidyl inositol (V_1). Recent evidence suggests that certain renal epithelial tissue (LLC-PK_1 cells) possess both V_1 receptors (Garg et al., 1988) and V_2 receptors (Lester et al., 1985).

The basolateral membrane preparation yielded a 3.25-fold increase in Na^+K^+-ATPase activity with respect to the P_2 pellet and a 10-fold increase compared to the homogenate. Vasopressin binding sites per unit protein doubled in the basolateral preparation over that in the P_2 fraction. The discrepancy may be explained in terms that while basolateral membranes may be enriched, not all of the membranes may possess AVP receptors due to their heterogeneous distribution along the renal tubule (Morel et al., 1987). Scatchard analysis revealed that a single class of high affinity AVP receptors is present on these basolateral membranes. Vasopressin binding was rapid, reversible, specific and displayed a pharmacological profile characteristic of V_2 (adenylate cyclase-coupled) vasopressin receptors. The maximal binding capacity determined in the present study (~220 fmol/mg P) is somewhat less than the value of 300 fmol/mg P obtained by Rajerison et al. (1977) but is in close agreement with the value of 228 fmol/mg P reported by Cornett and Dorsa (1985). This variation is not entirely surprising, however, given the differences in membrane preparation and other variations in the protocol.

In summary, these data demonstrate the presence of high affinity receptors for arginine^8-vasopressin on basolateral membranes prepared by differential and density gradient centrifugation from rat renal tubular epithelia. These data are consistent with and

complement other studies that have examined the vasopressin-sensitive adenylate cyclase system in the kidney and its coupling to V_2-type receptors (Roy et al., 1975; Rajerison et al., 1977; Butlen et al., 1978).

CHAPTER 3
DEHYDRATION-INDUCED DOWNREGULATION OF RENAL TUBULAR VASOPRESSIN RECEPTORS

Introduction

Several endocrine systems act to maintain fluid and electrolyte balance. Alterations of fluid homeostasis influence plasma levels of a number of hormones. This is particularly evident during dehydration. This condition leads to elevated circulating levels of vasopressin (Aisenbrey et al., 1981; Woods and Johnston, 1983), angiotensin II (Di Nicolantonio and Mendelsohn, 1986) and endogenous opiate peptides (Mata et al., 1977). Plasma levels of atrial natriuretic peptide on the other hand tend to be lower, which is consistent with the physiological role of this hormone (Ogawa et al., 1987). In turn, these hormones act upon their respective receptors in proportion to the response required to re-establish volume homeostasis. Paramount to this task is the action of arginine vasopressin, exerting its well-known antidiuretic and vasoconstrictive effects, through interactions with V_2 and V_1 receptors, respectively.

Peptide hormone receptors can be regulated by the concentration of hormone to which they are exposed. This can often lead to a condition described as "downregulation." This phenomenon results from withdrawal of cell surface membrane receptors by an internalization process. Reduced cellular sensitivity to hormonal stimulation can also occur by an uncoupling of the existing receptors from the signal-transducing effector system. The most well-

documented examples of these types of regulation come from the elegant studies by Lefkowitz et al. (1984) involving β-adrenergic receptors. His group has demonstrated examples of "homologous" regulation by receptor-specific agonists such as isoproterenol and "heterologous" regulation, where stimulation by a drug or hormone leads to a change in sensitivity to another unrelated drug or hormone. They found that thyroid status could have a profound effect on both the number and coupling of α-adrenergic and β-adrenergic receptors. Presumably, this control of adrenergic receptors was exerted at the level of gene transcription. Adrenergic receptors are by no means unique in terms of their susceptibility to regulation. Insulin receptors too, undergo homologous regulation by insulin (Gavin et al., 1974). Insulin receptors also undergo heterologous regulation, under the action of β-adrenergic agents (Pessin et al., 1983). Additional examples and more insight into the phenomena of receptor regulation may be gained from the recent reviews by Roth and Taylor (1982) and Poste (1984).

The cellular sensitivity to vasopressin can be drastically reduced following chronic stimulation by the hormone. When the isolated toad bladder, an epithelial model for the mammalian renal tubule, is stimulated repetitively with high concentrations of the neuro-hypophyseal hormone, the osmotic water flow across the bladder diminishes progressively (Eggena, 1981). Renal medullary plasma membranes incubated with high concentrations of vasopressin display reduced hormone-sensitive adenylate cyclase activity (Roy et al., 1976). Furthermore, vasopressin-elicited desensitization of adenylate cyclase activity has been shown in renal membranes prepared from

rats injected with pharmacological doses of vasopressin (Rajerison et al., 1977). AVP-induced downregulation of vasopressin receptors and a concomitant desensitization of vasopressin-sensitive adenylate cyclase activity has been demonstrated in a renal epithelial cell line (Lester et al., 1985).

Thus, vasopressin can affect the cellular sensitivity of its target tissue by regulating its own receptor. Because dehydration is a condition characterized by high circulating levels of AVP, the present study was conducted in order to determine whether vasopressin receptors are commensurately regulated under conditions of water deprivation.

Materials and Methods

Animals

Male Sprague-Dawley rats (n=6 per group) weighing 200-250g were obtained from the Animal Resource Facility at the University of Florida. Rats were housed individually in 20x18x24 cm wire mesh cages, exposed to a 12:12 hour light:dark cycle, provided a standard Purina rat chow diet and allowed free access to tap water. The dehydrated experimental group was deprived of water for 72 hours prior to sacrifice.

Chemicals

Tritiated 8-L-arginine [phenylalanine-3,4,5-^{3}H]-AVP ([^{3}H]-AVP) used in receptor binding studies was purchased from New England Nuclear Corp. (Boston) with a specific activity of 70 Ci/mmol. Iodinated vasopressin (^{125}I-AVP), specific activity of 2200 Ci/mmol, was also obtained from New England Nuclear for use in the vasopressin radioimmunoassay (RIA). AVP antibody was purchased

from Amersham (Arlington Hts., IL) while AVP standard was obtained from Peninsula Labs (Belmont, CA). Other reagents were obtained from Sigma, unless otherwise indicated.

Membrane Preparation and Plasma Collection

Animals were sacrificed by decapitation and trunk blood was collected. A 100 μl sample was obtained separately for the determination of plasma osmolality while the remainder was collected in chilled tubes containing 0.3 M EDTA (50 μl/ml) for the determination of plasma AVP concentration. Blood samples were centrifuged at 3,000 x g for 10 minutes at 4°C, the plasma removed and stored at -20°C. Renal basolateral membranes were prepared by modifying the density gradient centrifugation method of Boumendil-Podevin and Podevin (1983), according the description provided in Chapter 2.

Vasopressin Receptor Assay

Membranes, corresponding to 100-200 μg protein were suspended in 300 μl of 100 mM Tris-HCl buffer containing 5 mM $MgCl_2$, 1 mM EGTA and 0.1% BSA, pH 7.3 at 20°C. Triplicate samples were incubated for 45 minutes at 20°C with concentrations of ^{3}H-AVP ranging from 50 pM to 5,000 pM. Non-specific binding was determined in the presence of 5 μM unlabelled AVP. Further details of the receptor binding protocol are described in Chapter 2.

RIA for Vasopressin

Plasma samples for the determination of AVP were thawed and purified using SepPak C_{18} cartridges (Waters). Briefly, the purification process is as follows: Cartridges are pre-wetted with 5 ml methanol followed by 5 ml 1% trifluroacetic acid (TFA). A 1 ml

plasma sample is acidified with 1% TFA and applied to the column dropwise. The cartridge was rinsed with 5 ml 1% NaCl/1% TFA, then AVP was eluted with 4 ml methanol:H_2O:TFA (80:19:1). The samples were evaporated under a light stream of air then reconstituted in 0.5 ml of assay buffer (50 mM phosphate buffer, pH 7.4 containing 0.2% bovine serum albumin and 10 mM EDTA). The RIA was carried out by incubating 100 μl samples or known amounts of standard (0.1-20 pg AVP) with 25 μl AVP antiserum and 25 μl ^{125}I-AVP (corresponding to 3,000 cpm) for 24 hours at 4°C. Free ligand was sequestered by the addition of dextran-coated charcoal and centrifugation at 2,000 x g for 15 minutes. The supernatant containing the antibody-bound ligand was aspirated and counted in a Beckman 5500 gamma counter at an efficiency of 79% for ^{125}I. Values are expressed as pg AVP. Cross-reactivity with related peptides is <1%. Recovery of AVP for the extraction procedure was determined to be >90%. Sensitivity of the assay was 0.15 pg per tube.

Plasma Osmolality

Plasma osmolality was determined using a Westcor model 5500C vapor pressure osmometer. Data are expressed as mosmols/kg H_2O.

Analysis

Data from saturation and competition experiments were analyzed using iterative curve fitting programs. Data are reported as the mean ± S.E. Statistical significance of the data was assessed using one-way analysis of variance and Student's t-test. A level of $p<.05$ was considered to be significant.

Results

The state of hydration of the rats was evaluated by comparing the urine ouput and osmolality, plasma osmolality and plasma vasopressin concentration among the two groups. Urine output in the normally hydrated control group was 10.6±1.1 ml/24 hr while that of the dehydrated group was 1.9±0.8 ml/24 hr ($p<.05$ vs. control) (Figure 3-1A). Urine osmolality was significantly elevated among the dehydrated animals (2789±169 mosmol/kg) when compared to control values (1447±225 mosmols/kg) ($p<.05$) (Figure 3-1B). Similarly, plasma osmolality was raised from control values of 288 ± 0.8 mosmol/kg to 299 ± 1.7 mosmol/kg among the water deprived group ($p<.05$) (Figure 3-2A). Vasopressin values were determined from the standard curve shown in Figure 3-3. In response to the heightened osmolality of the extracellular fluid, plasma vasopressin levels were commensurately elevated. AVP concentrations were raised 11.5-fold, from a value of 0.21±0.02 pg/ml in the plasma of the control animals to 2.4±0.3 pg/ml in the plasma of the dehydrated animals ($p<.05$) (Figure 3-2B).

^{3}H-vasopressin binding was determined in renal tubular epithelial basolateral membranes prepared from the kidneys of the control and dehydrated animals. A representative saturation isotherm for ^{3}H-AVP binding is depicted in Figure 3-4. Transformation of the data for Scatchard analysis is represented in Figure 3-5. Analysis of the data between the two groups indicates a significant reduction in the number of vasopressin receptors on the membranes from the dehydrated animals (Control B_{max} = 184±15 fmol/mg protein vs. Dehydrated B_{max} = 114±2.5 fmol/mg protein) ($p<.01$). No significant difference was observed in the affinity of the receptor between the

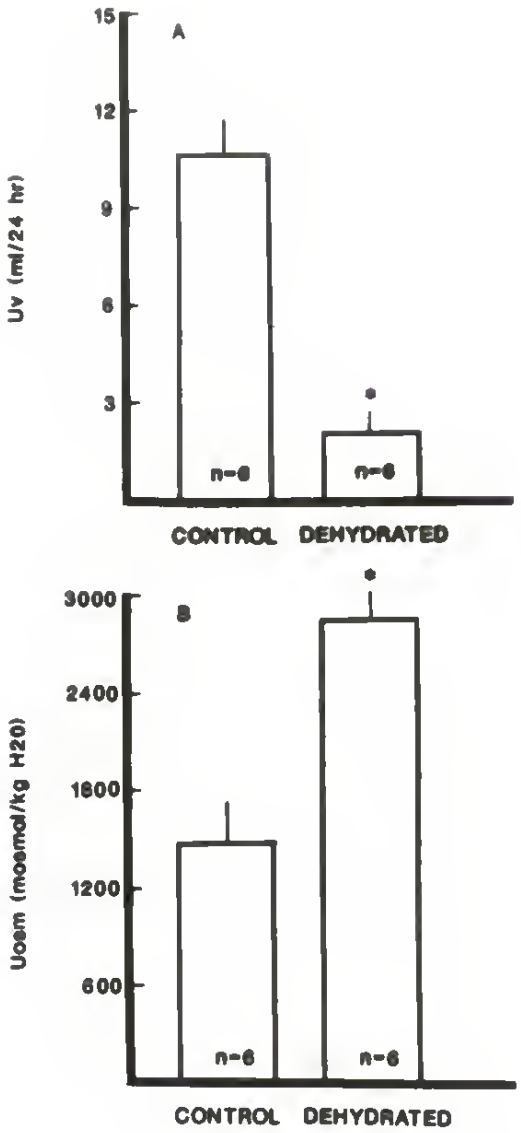

Figure 3-1. Urine output and urine osmolality in control and dehydrated rats. A: urine output (Uv). B: urine osmolality (Uosm). Osmolality determined by vapor pressure osmometry. Error bars represent S.E.M. *$p<.05$ vs. control.

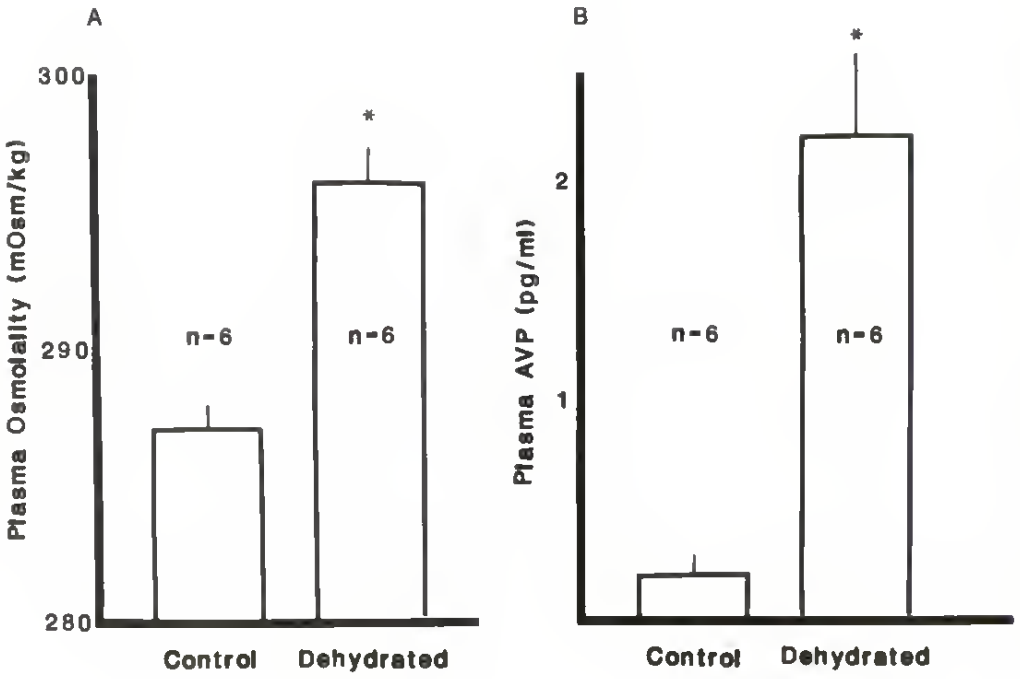

Figure 3-2. Plasma osmolality and plasma vasopressin concentrations in control and dehydrated rats. A: Plasma osmolality, determined by vapor pressure osmometry. B: Plasma AVP determined by RIA. Error bars represent S.E.M. *p<.05 vs. control. AVP, arginine vasopressin; RIA, radioimmunoassay.

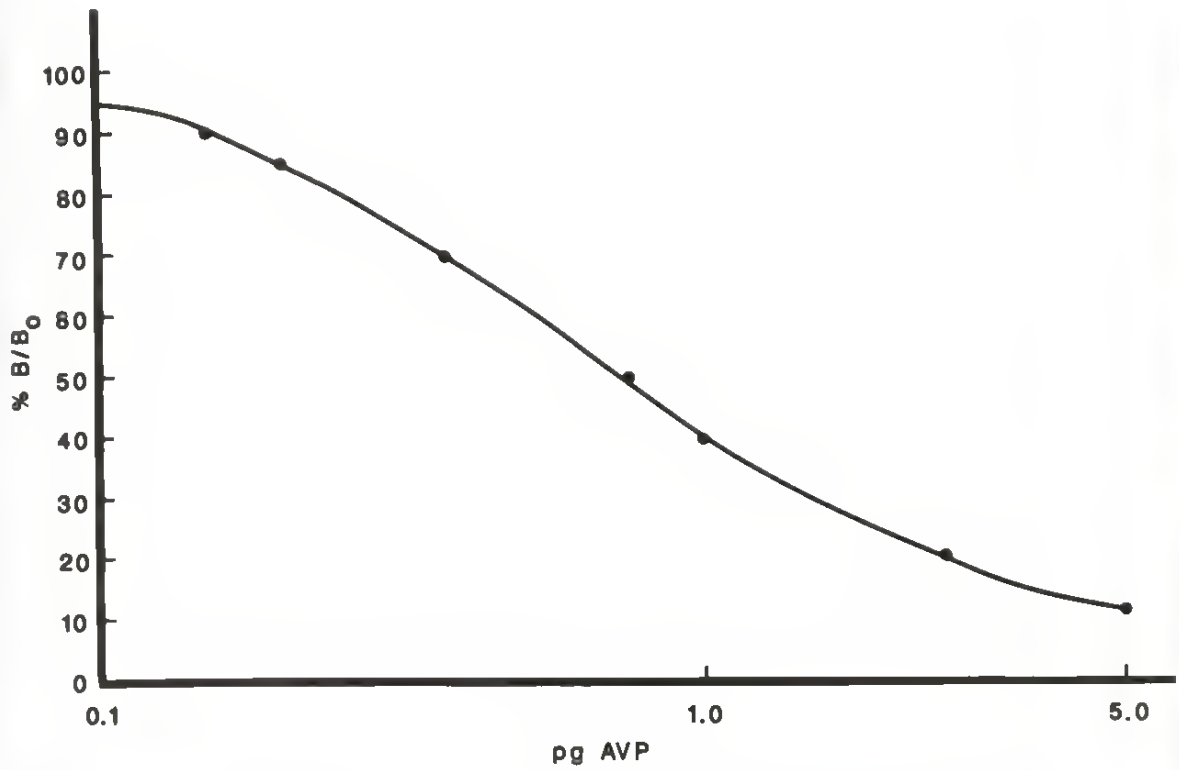

Figure 3-3. Standard curve for arginine vasopressin (AVP). Values determined by radioimmunoassay. Details described in "Methods" section.

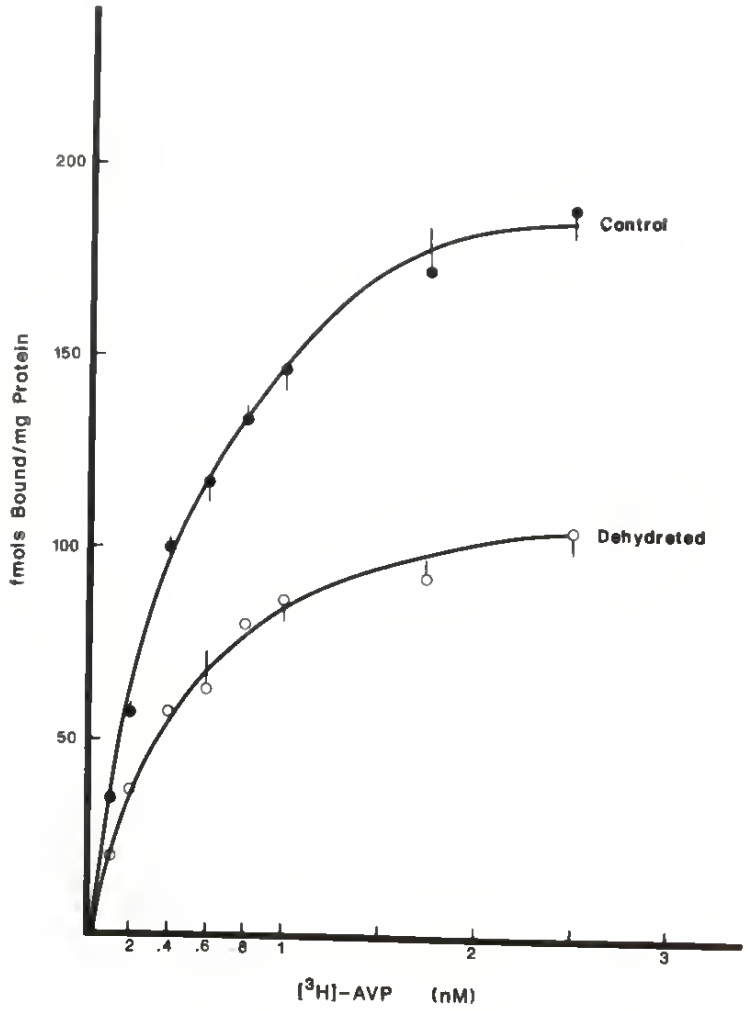

Figure 3-4. Saturation isotherms of ^{3}H-vasopressin (^{3}H-AVP) binding. Binding determined in basolateral membranes prepared from kidneys of water-replete and water-deprived (72 hr) rats. Details of binding protocol described in "Methods" section of Chapter 2. Error bars represent S.E.M. of triplicate determinations.

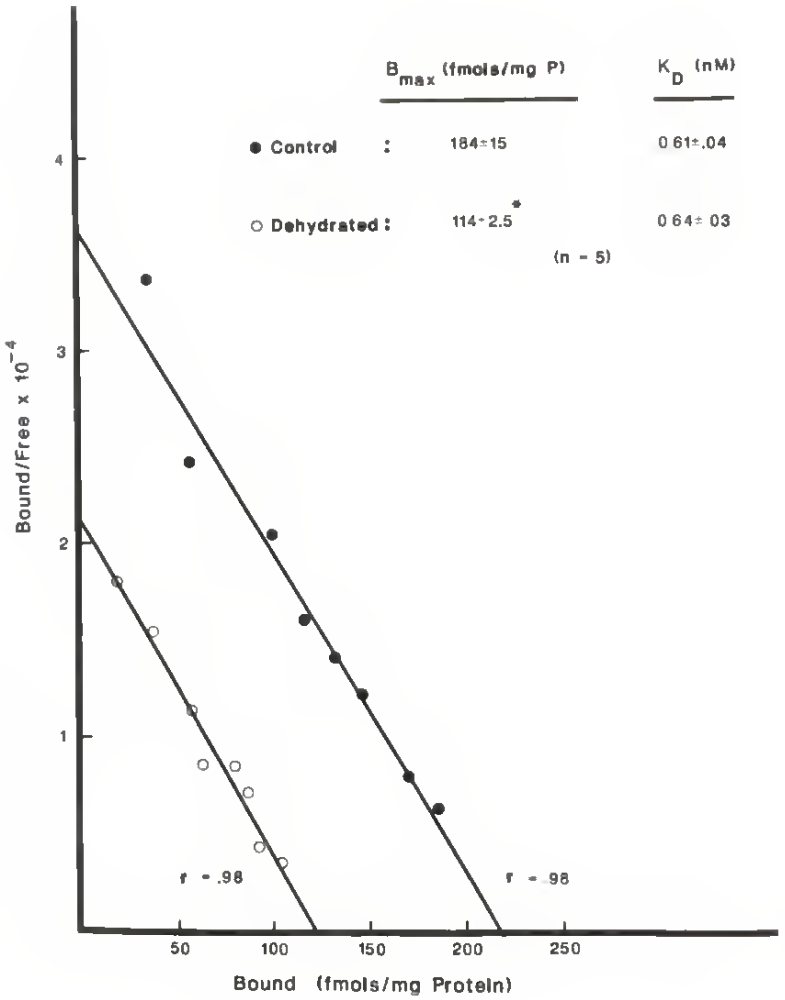

Figure 3-5. Scatchard analyses of ^{3}H-vasopressin binding. Binding determined in basolateral membranes prepared from kidneys of water-replete and water-deprived (72 hr) rats. Analyses of data in Figure 3-4. *$p<.05$ vs. control.

two groups (Control K_D = 0.61±.04 nM vs. Dehydrated K_D = 0.64±.03 nM).

Discussion

Alterations in fluid balance can affect not only the hormones themselves which participate in its regulation but also the receptors which mediate the biological responses of the hormones. Thus, dehydration causes a reduction in κ-opiate receptor binding in the neurohypophysis (Brady and Herkenham, 1987) and a decrease in angiotensin II binding in the adrenal medulla (Hwang et al., 1986). Alternatively, receptors for atrial natriuretic factor are increased in the kidney and adrenal gland, in response to reduced circulating levels of the hormone (Lynch et al., 1986).

These data in the present study show that V_2-type vasopressin receptors located on renal tubular epithelial basolateral membranes are modulated when fluid homeostasis is compromised by dehydration. A significant reduction in the number of vasopressin receptors (B_{max}) was noted in the renal tubular basolateral membranes prepared from the water-deprived animals. There was no difference between the two groups with respect to the affinity (K_D) of the receptor for the hormone. Water deprivation for 72 hours significantly elevated plasma AVP concentrations 11.5-fold. The magnitude of this increase is comparable to the response observed by Woods and Johnston (1983) in rats dehydrated for a similar period of time. Thus, it would appear that the receptors are sensitive to the circulating levels of vasopressin. This is supported by the study of Shewey and Dorsa (1986) who reported finding no difference in the level of ^{3}H-AVP binding in renal medullary membranes prepared from heterozygous

Brattleboro rats and their chronically dehydrated homozygous counterparts, which are unable to synthesize AVP. This would suggest that the reduction in vasopressin receptor density observed in the present study is attributable to the elevated plasma levels of vasopressin, rather than some nonspecific effect of dehydration.

Homologous downregulation of vasopressin receptors has been reported in a renal epithelial cell line (Lester et al., 1985) and in the toad urinary bladder (Eggena and Ma, 1986), following treatment with exogenous vasopressin. The phenomenon of downregulation results from an increased rate of internalization when the hormone concentration has been greatly enhanced (Catt et al., 1979). The "anatomical correlate" of downregulation has been described by Kirk (1987). He has elegantly shown by morphometric analysis that retrieval or internalization of the basolateral membrane occurs in the cells of the rabbit cortical collecting tubule when induced by a vasopressin-stimulated transcellular water flow. He suggests that membrane internalization could reduce the sensitivity of the cortical collecting tubule cells to vasopressin if AVP receptors are present on the retrieved membrane. While not addressed in the present study, the issue of adenylate cyclase responsiveness has been examined in conjunction with the downregulation of AVP receptors. Rajerison et al. (1977) showed in the rat that vasopressin-sensitive adenylate cyclase activity in membrane preparations from the rat kidney medulla is greatly reduced after the infusion of pharmacological doses of AVP to the animals.

In the present study, it would seem odd that an animal in dire need of water conservation would lose AVP receptors as the need

became greater. An explanation for this may be that the kidney possesses "spare" receptors (Ariens et al., 1960). Baddouri et al. (1984) noted a downregulation of vasopressin receptors in the kidney of the water deprived jerboa, Jaculus orientalis. Water-deprived animals in that study displayed a five-fold elevation in plasma AVP levels and a 36% reduction in ^{3}H-lysine vasopressin binding to renal medullary membranes. Despite reduced vasopressin binding, the kidneys of some of these desert rodents still retained the capacity to significantly concentrate urine under these conditions. The latter finding suggests that "spare" receptors may have been involved in the regulatory phenomenon. A receptor reserve has also been noted in other adenylate cyclase-linked tissues as well (Flores et al., 1974). Alternatively, reduced binding without a decrement in biological responsiveness may occur if those receptors which were downregulated are "silent", or not coupled to an effector system. Maack et al. (1987) have recently shown that the overwhelming majority (>90%) of receptors for atrial natriuretic factor that are present in the kidney are biologically silent. That is to say, they do not mediate any of the known renal effects of the hormone. The authors speculate that these silent receptors may serve as "peripheral storage clearance binding sites, acting as a hormonal buffer system to modulate plasma levels of atrial natriuretic factor". The physiological significance of silent or spare receptors and their regulation remains to be explored.

In summary, the present study shows that V_2 vasopressin receptors are differentially expressed in basolateral membrane preparations from the kidneys of water-replete and water-deprived

rats. Scatchard analyses indicate that the difference in binding is due to a reduction of receptor numbers on the membranes prepared from the dehydrated animals. These results show that AVP receptor downregulation can occur in response to physiologically elevated levels of circulating vasopressin. Such a condition could ultimately affect the antidiuretic action of the hormone.

CHAPTER 4
REGULATION OF VASOPRESSIN BINDING IN MINERALOCORTICOID-DEPENDENT HYPERTENSION

Introduction

The role of vasopressin in the etiology of hypertension has received considerable attention, much of it being reviewed recently by McNeill (1983) and Johnston (1985). The pressor action of vasopressin was the first physiological response attributed to this posterior pituitary substance (Oliver and Schafer, 1895) and the vasoconstrictive properties of the hormone are now well-documented both in vivo (Liard, 1984) and in vitro (Altura and Altura, 1984). One line of evidence which points to a relationship between vasopressin and high blood pressure is that circulating levels of vasopressin are elevated in several models of hypertension. Crofton et al. (1978) noted higher plasma vasopressin concentrations in the spontaneously hypertensive rat. Similar observations were noted in renovascular (one kidney,one clip) (Pullan et al., 1978) and volume-expanded (DOCA-salt) (Mohring et al., 1977; Crofton et al., 1979) models of hypertension. While the circulating levels of vasopressin are significantly increased in all of the above studies, the plasma concentrations are, nonetheless, below the threshold for pressor activity. This discrepancy can be resolved, however, by invoking increased vascular reactivity toward vasopressin. Such an enhanced pressor sensitivity to AVP has been noted in the SHR (Crofton et al., 1978) and in DOCA-salt hypertensive rats (Crofton et al., 1980).

Added support for the involvement of vasopressin in the pathogenesis of DOCA-salt hypertension comes from the study of Friedman et al. (1960). These investigators noted that the administration of AVP accelerated the development of DOCA-salt hypertension. The most telling piece of evidence however, which implicated vasopressin in DOCA-salt hypertension was the finding of Crofton et al. (1979) who reported that Brattleboro rats, which are unable to synthesize AVP, failed to develop DOCA-salt hypertension.

The development of selective antagonists to the pressor and antidiuretic effects of vasopressin greatly facilitated our understanding of the involvement of vasopressin in hypertension. Crofton et al. (1979) found that the administration of a selective V_1 (vascular) antagonist caused an acute fall in blood pressure in DOCA-salt hypertensive rats. Similarly, Mohring et al. (1977) were able to lower the blood pressure by the injection of a highly specific vasopressin antibody. Hofbauer et al. (1984) attenuated the development of DOCA-salt hypertension by the simultaneous infusion of V_1 antagonists. Collectively, these studies suggest that vasopressin may contribute to the development of hypertension by virtue of its vasoconstrictive properties. Conversely, other investigators (Rascher et al., 1983; Yamamoto et al., 1984; Filep et al., 1987) have been unable to reduce the high blood pressure associated with DOCA-salt hypertension by the administration of V_1 blockers.

A renal locus in the pathogenesis of DOCA-salt hypertension has been largely overlooked until recently. Saito and Yajima (1982) observed that when Brattleboro rats were treated with DDAVP, a

selective tubular (V_2) agonist, DOCA-salt hypertension developed. A similar phenomenon was observed by Woods and Johnston (1981), using renovascular models of hypertension in Brattleboro rats. Whereas Hofbauer et al. (1984) attenuated the development of DOCA-salt hypertension with V_1 blockers as noted above, the simultaneous infusion of a combined V_1/V_2 antagonist completely abolished the rise in blood pressure. These studies suggest a tubular site of action for vasopressin in the development of DOCA-salt hypertension.

The role of the kidney in the pathogenesis of hypertension has been a major focus of Guyton and his colleagues (Guyton et al., 1972). The action of vasopressin in this context was underscored in the findings of Manning et al. (1979) and Cowley et al. (1984b) who reported that subpressor doses of vasopressin can contribute to a rise in arterial pressure, which is dependent on the fluid-retaining properties of the hormone. In addition, it has been shown that vasopressin stimulates sodium reabsorption in the medullary thick ascending limb of the mouse (Hebert et al., 1981a) and the cortical collecting tubule of the rat (Reif et al., 1986). The latter authors also showed that the effects of vasopressin on sodium reabsorption are augmented in DOCA-treated rats.

Thus, it appears that there is an interaction between vasopressin and mineralocorticoids at the level of the renal tubule. Increased activity of the salt and fluid transport processes would lend itself to a volume-expanded state, with the potential for raising arterial pressure. The question of how vasopressin activity is increased in DOCA-treated animals remains unanswered. This study will address the question at the cellular level. Vasopressin receptors and

vasopressin-stimulated adenylate cyclase activity will be measured in basolateral membranes prepared from renal tubular epithelia in normotensive and DOCA-salt hypertensive rats.

Materials and Methods

Chemicals

^{3}H-argininie-vasopressin (^{3}H-AVP), ^{125}I-arginine-vasopressin (^{125}I-AVP) and ^{3}H-3′,5′-cyclic adenosine monophosphate (^{3}H-cAMP) were purchased from New England Nuclear Corporation (Boston). Peptides, cAMP, protein kinase, desoxycorticosterone acetate (DOCA), protein kinase, and the regenerating reagents for adenylate cyclase stimulation (theophylline, creatine phosphokinase, phosphocreatinine, ATP and GTP) were all obtained from Sigma Chemical Company (St. Louis). Vasopresssin antibody was purchased from Amersham (Arlington Heights, IL) and Liquiscint liquid scintillation fluid from National Diagnostics (New Jersey).

Animals

Thirty male Sprague Dawley rats weighing 175-225 g were obtained from the Animal Resource facility at the University of Florida. Rats were housed in a climate-controlled room and caged individually in 20x18x24 cm wire mesh cages. They were provided a standard Purina rat chow diet and allowed free access to tap water. The rats were divided into five groups containing six rats per group. Under chloral hydrate anesthesia (0.4 g/kg body weight, i.p.), animals in Group I were implanted subcutaneously with two silastic capsules, 5 cm long, containing DOCA. They were unilaterally nephrectomized using a retroperitoneal approach and provided with a 1% NaCl drinking solution in lieu of water. Group II animals received 1% NaCl

drinking solution and was unilaterally nephrectomized according to the procedures described above, but received no DOCA implants. Animals in Group III received DOCA implants, tap water to drink and their kidneys remained intact. Those in Group IV were implanted with DOCA capsules, received 1% NaCl to drink, but their kidneys were left intact. Animals in Group V received no special treatment and were provided with tap water to drink. Blood pressures were recorded by tail cuff plethysmography prior to surgery and once weekly, thereafter. Values are expressed as mm Hg.

Membrane Preparation and Plasma Collection

After four weeks of treatment, animals were sacrificed by decapitation. Trunk blood was collected in chilled tubes containing 0.3 M EDTA (50 μl/ml blood) for the determination of plasma vasopressin concentration. Blood samples were processed according to the procedure described in Chapter 3. Renal basolateral membranes were prepared by modifying the density gradient centrifugation method of Boumendil-Podevin and Podevin (1983), according to the description in Chapter 2. For vasopressin receptor binding assays, membranes were stored in liquid N_2 until use. For adenylate cyclase measurements, membranes were used immediately.

Vasopressin Receptor Assay

Binding studies were conducted according to the procedure described in Chapter Two. Briefly, membrane samples were incubated in triplicate at 20°C for 45 minutes with concentrations of ^{3}H-AVP ranging from 50 pM to 2,500 pM. Non-specific binding was determined in the presence of 2.5 μM unlabelled AVP.

Vasopressin Radioimmunoassay

Plasma vasopressin concentrations were determined according to the method described in Chapter 3.

Adenylate Cyclase Stimulation

Basal and vasopressin-stimulated adenylate cyclase activity in renal basolateral membranes was performed according to the following protocol: Basal samples (membranes corresponding to 100 μg protein) were suspended in a total volume of 150 μl of 50 mM Tris buffer, pH 7.4, containing 138 μg ATP, 39 μg GTP, 150 μg $MgCl_2$, 38 μg EGTA, 150 μg BSA, and a regenerating system consisting of 228 μg theophylline, 60 μg creatine phosphokinase and 100 μg phosphocreatine. Agonist-stimulated samples were prepared as above but were incubated in the presence of AVP ranging in concentration from 0.1 nM to 10 μM. Triplicate samples were incubated at 37°C for 10 minutes. The reaction was stopped by the addition of 300 μl of 100 mM Tris buffer containing 5 mM EDTA, pH 7. The samples were boiled for 5 minutes and then centrifuged at 3,000 rpm for 5 minutes. Supernatants were removed at stored at -20°C for subsequent determination of cAMP formation.

cAMP Determination

The cellular cAMP content was determined by the competitive protein binding assay as modified by Baker et al. (1986). Samples for cAMP determination were reconstituted in 200 μl of 25 mM Tris buffer, pH 7.0. 10 μl of each sample were incubated at 4°C for 60 minutes in a total volume of 200 μl Tris buffer containing 25,000 cpm of ^{3}H-cAMP, 42 μg BSA, 280 μg theophylline and the reaction initiated by the addition of 24 μg of cAMP-dependent protein kinase.

At the end of the incubation, 70 μl of a hydroxyapatite suspension (50% w/v) was added to each tube, vortexed and allowed to sit for six minutes. The contents of the tube were then filtered under vacuum through Whatman GF/C filters, then washed with 12 ml of 10 mM Tris buffer at pH 7.0. Filters were removed and transferred to scintillation vials containing 1 ml of 0.3N HCl. After the hydroxyapatite had dissolved (~10 min), 9 ml of liquid scintillation fluid (Liquiscint) was added and each vial counted. Non-specific binding was determined in the presence of 10^{-5}M cAMP. The amount of cAMP present in each sample was calculated from a standard curve determined with known amounts of unlabeled cAMP.

Analysis

Data from saturation studies were analyzed using iterative curve fitting programs. Statistical significance was assessed by one-way analysis of variance. The post-hoc analysis between treatment pairs was performed by Duncan's method. A "p" value of 0.05 or less was considered an acceptable level of significance.

Results

The combination of desoxycorticosterone acetate treatment and increased sodium intake precipitates the development of the well-characterized syndrome of DOCA-salt hypertension. Figure 4-1 depicts the rapid development of high blood pressure in rats administered the mineralocorticoid and offered 1% NaCl to drink. Those animals in group I with a reduced renal mass became hypertensive most rapidly and attained the highest arterial pressure (176±5 mm Hg). Even those animals with intact kidneys but provided a similar regimen (Group IV) became hypertensive (154±8 mm Hg)

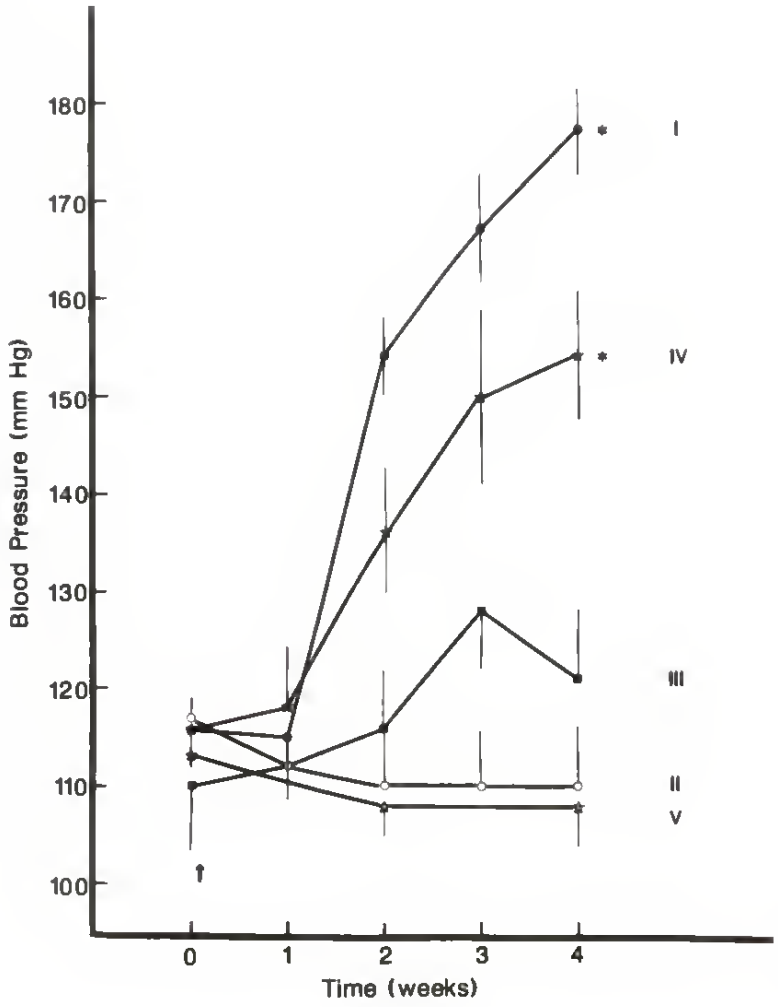

Figure 4-1. Arterial pressure in control and DOCA-treated rats. Blood pressures were determined prior to experiment and once per week thereafter by tail cuff plethysmography. Arrow indicates start of treatment. Error bars represent S.E.M. (n = 6 per group except V, where n = 4). *p<.05 vs. V and II. See "Methods" section for key to group designations.

after only 4 weeks of treatment. This contrasts to the animals in group III who received only DOCA and displayed an arterial pressure of 121±7 mm Hg. This value was not significantly different from the values observed in animals in Groups V (108±8 mm Hg) and II (110±6 mm Hg), receiving no treatment or 1% NaCl and unilateral nephrectomy, respectively.

^{3}H-vasopressin binding in renal epithelial basolateral membranes prepared from the DOCA-salt hypertensive rats (Group I) and their appropriate controls (Group II) revealed a higher level of binding in membranes from the hypertensive group (Figure 4-2). As depicted in Figure 4-3, Scatchard analysis of these data indicate that this higher level of binding is due to an increased number of binding sites (Group I = 307±29 fmoles/mg protein vs. Group II = 179±17 fmoles/mg protein) [$p<.05$], rather than a change in affinity (Group I = 0.59±.11 nM vs. Group II = 0.54±.09 nM) [$p>.05$]. This represents a 71% increase over control values. Table 4-I summarizes the binding data for the other experimental groups. It can been noted that a higher B_{max} is also evident for the hypertensive animals in Group IV (281±33 fmoles/mg protein). This too, is significantly higher than the values for Group II ($p<.05$). Furthermore, there were no significant differences between the values in Group II and the untreated animals in Group V (B_{max} = 196±23 fmoles/mg protein; K_D = 0.67±.12) [$p>.05$]. Binding (B_{max} = 191±14 fmoles/mg protein; K_D = 0.63±.04) in membranes prepared from the animals receiving DOCA only (Group III) was not significantly different from either of the control groups (II or V).

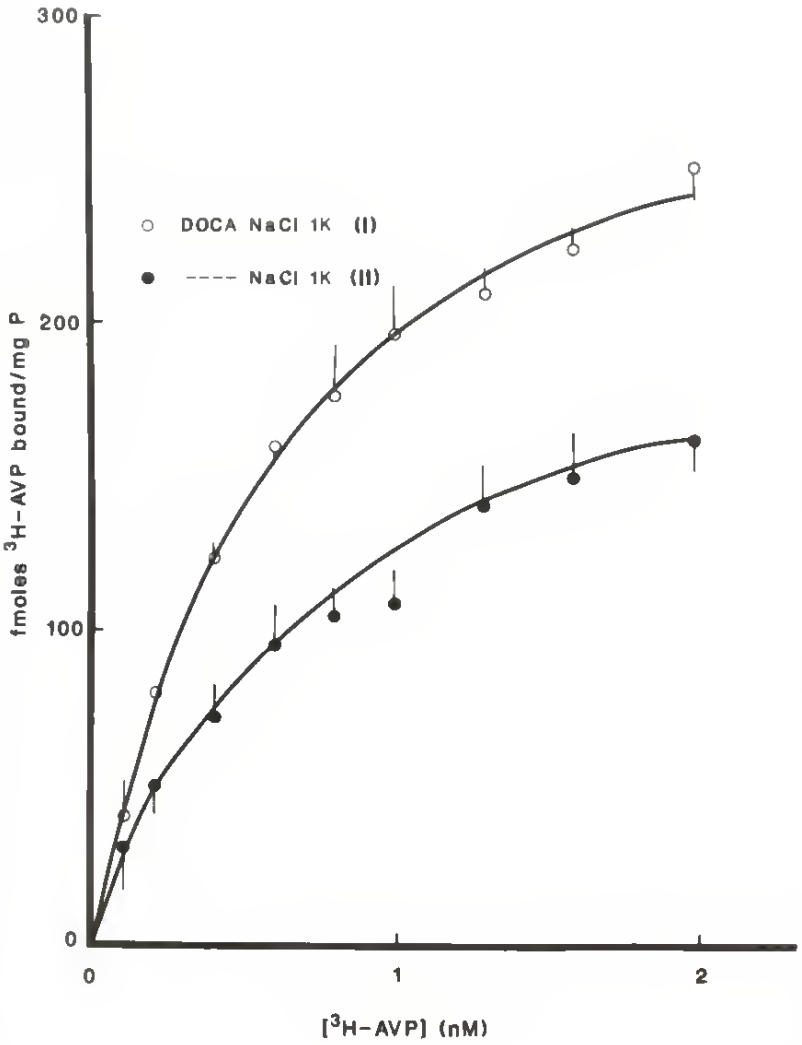

Figure 4-2. Saturation isotherms of ^{3}H-vasopressin (^{3}H-AVP) binding. Binding determined in basolateral membranes prepared from kidneys of DOCA-salt hypertensive rats and normotensive sham-operated control rats. Details of binding protocol described in "Methods" section of Chapter 2. Error bars represent S.E.M. of triplicate determinations. DOCA, desoxycorticosterone acetate; Na, 1% NaCl; 1K, one kidney.

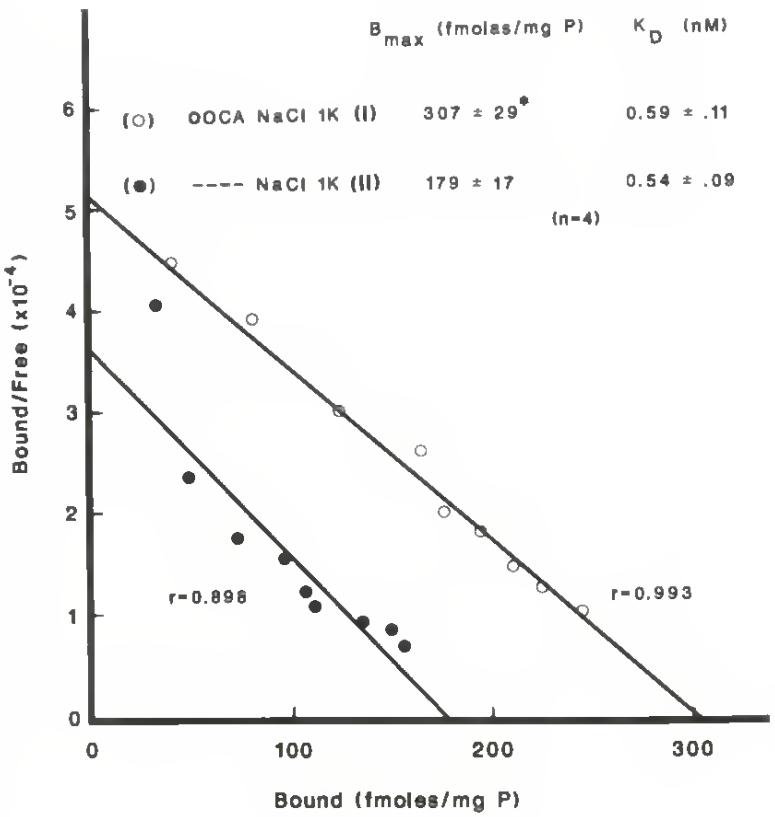

Figure 4-3. Scatchard analyses of ^{3}H-vasopressin binding. Binding determined in basolateral membranes prepared from kidneys of DOCA-salt hypertensive rats and normotensive sham-operated control rats. Analyses of data in Figure 4-2. *p<.05 vs. control. Abbreviations are as in Figure 4-2.

TABLE 4-1.
VASOPRESSIN BINDING IN DOCA-TREATED RATS.

Treatment Group	B_{max} (fmoles/mg P)	K_D (nM)	n
I	307 ± 29*	0.59 ± .11	4
II	179 ± 17	0.54 ± .09	4
III	191 ± 14	0.63 ± .04	3
IV	281 ± 33*	0.62 ± .14	2
V	196 ± 23	0.67 ± .12	3

* $p < .05$ vs. Groups II and V

Basal and vasopressin-stimulated cyclic AMP formation was measured in freshly prepared membranes from the various groups. The values were derived from the standard curve depicted in Figure 4-4. Figure 4-5 represents the production of cAMP in membranes prepared from animals in the DOCA-salt hypertensive group (I) and the corresponding control group (II). Whereas basal values did not differ from each another, membranes from the hypertensive animals were stimulated to a greater degree than membranes from the control group. The enhanced sensitivity to vasopressin is evidenced by a shift toward the left for the curve depicting Group I values. Subtracting basal values, the calculated ED_{50}'s for the hypertensive and control group are $2x10^{-10}$ M and $8.5x10^{-10}$ M, respectively. Table 4-2 summarizes the basal and vasopressin-stimulated cyclic AMP production in membranes of the other experimental groups. Basal values did not differ from one another but a greater level of stimulation and an enhanced sensitivity toward vasopressin were also noted in membranes from the hypertensive animals in Group IV.

Finally, plasma vasopressin levels were determined in the animals of the various groups and those data are presented in Table 4-3. Values for the non-hypertensive animals in Groups II and V ranged from 1.2±0.2 pg/ml to 1.8±0.2 pg/ml, respectively. Plasma vasopressin concentrations were significantly elevated among the hypertensive animals. Measured values in Groups I and IV were 6.1±0.8 pg/ml and 8.4±4.5 pg/ml, respectively. This represents a five-fold elevation in plasma vasopressin concentrations between the hypertensive and normotensive animals.

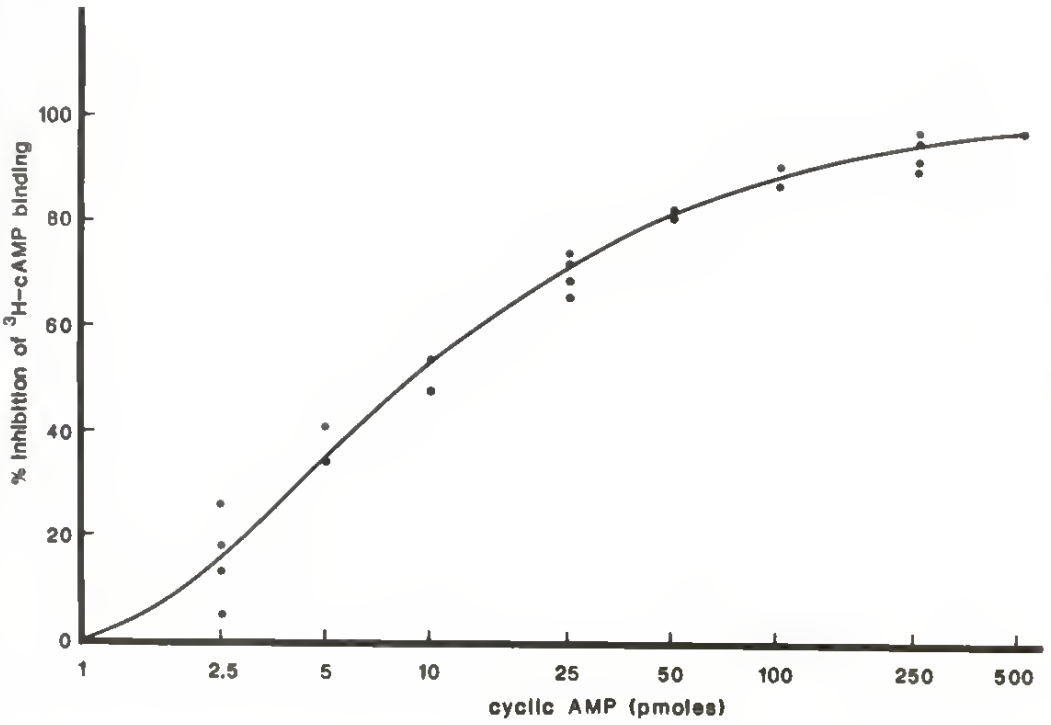

Figure 4-4. Standard curve for cyclic AMP. Values for cyclic AMP determined by competitive binding assay, using ^{3}H-cAMP. Details of procedure described in "Methods" section. cAMP, adenosine 3'5'-cyclic monophosphate.

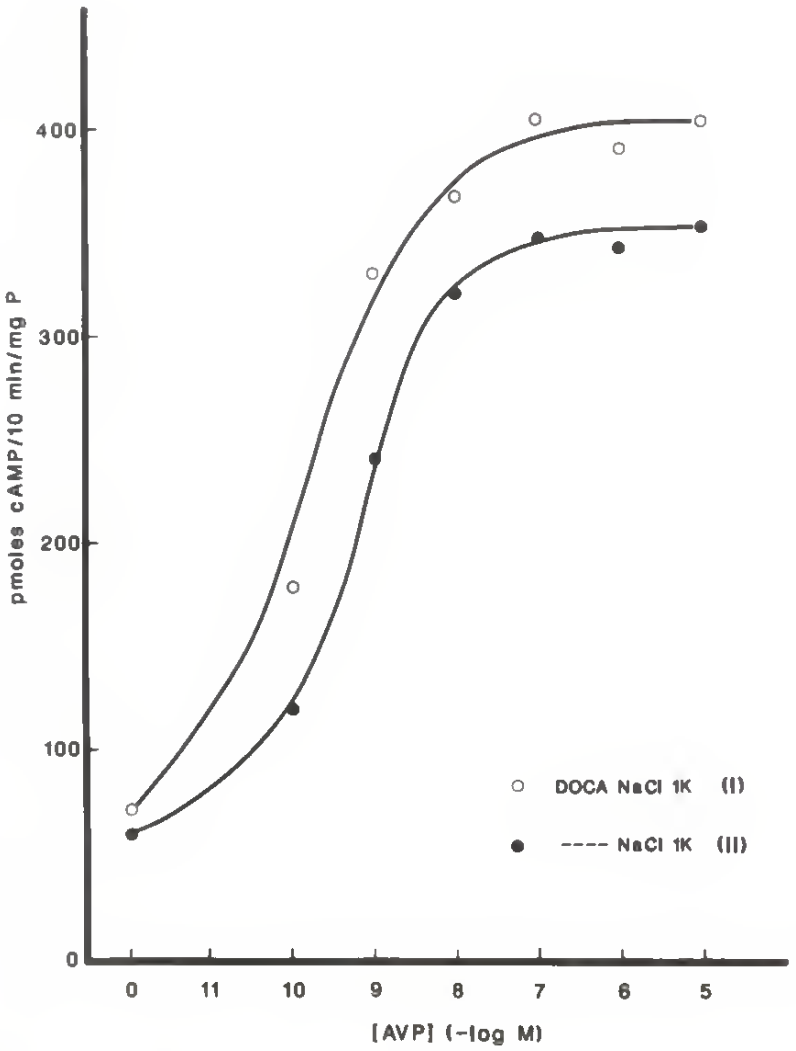

Figure 4-5. Basal and vasopressin-stimulated cyclic AMP formation. Cyclic AMP production by basolateral membranes prepared from DOCA-salt hypertensive rats and normotensive sham-operated control rats. Membrane preparation described in "Methods" section of Chapter 2. Procedures for the stimulation and determination of cyclic AMP described in "Methods" section. Abbreviations are as in Figure 4-2 except AVP, arginine vasopressin; cAMP, adenosine 3'5'-cyclic monophosphate.

TABLE 4-2.
VASOPRESSIN STIMULATED cAMP FORMATION IN DOCA SALT HYPERTENSIVE RATS

Group	Basal pmoles/10 min./mg P	Maximal Stimulation pmoles/10 min./mg P	IC_{50}
I	72 ± 14	410 ± 20	$2x10^{-10}M$
II	61 ± 12	355 ± 10	$8x10^{-10}M$
III	64 ± 07	360 ± 10	$7x10^{-10}M$
IV	65 ± 10	400 ± 12	$2.5x10^{-10}M$
V	58 ± 15	345 ± 15	$8.5x10^{-10}M$

TABLE 4-3.
PLASMA AVP LEVELS IN DOCA-SALT HYPERTENSIVE RATS

Group	[AVP] (pg/ml)
I	6.1 ± 0.8
II	1.8 ± 0.2
III	1.9 ± 0.3
IV	8.4 ± 4.5
V	1.2 ± 0.2

Discussion

The role of vasopressin in the development of DOCA-salt hypertension appears to be unequivocal. Blood pressure will not increase in this model unless vasopressin is present. This was demonstrated most elegantly by the use of Brattleboro rats, animals which are genetically incapable of synthesizing AVP (Crofton et al., 1979). What is unclear is the mechanism or mechanisms which underlie the etiology of the blood pressure increase. Various target tissues have been implicated to account for the hypertensive actions of vasopressin. A central site of action has been suggested by the study of Chen et al. (1986). These investigators reported heightened vasopressinergic activity in DOCA-salt hypertensive rats. This augmentation in hypothalamic release of vasopressin was exclusive of, and preceeded the well-known increase in peripheral AVP levels or elevation of blood pressure. The authors suggested that this alteration in central vasopressinergic activity may affect blood pressure by modulation of the cardiovascular centers in the brain. Studies with vasopressor antagonists suggest a vascular site of action. Crofton et al. (1979) reported that the acute administration of V_1 blockers lowered blood pressure in animals with established DOCA-salt hypertension. Hofbauer et al. (1984) found that the chronic administration of V_1 blockers during the DOCA-salt regimen attenuated the development and magnitude of hypertension associated with this model. Other studies however, tend to refute these findings (Rascher et al., 1983; Yamamoto et al., 1984; Filep et al., 1987).

A tubular site of action for vasopressin in the development of DOCA-salt hypertension is suggested by the study of Saito and Yajima (1982). Using Brattleboro rats, these investigators observed that DOCA-salt hypertension would develop following the chronic administration of DDAVP, a V_2-specific tubular agonist. These findings are consistent with the hypothesis of Cowley et al. (1984) that subpressor doses of vasopressin can contribute to a rise in arterial pressure, by virtue of the fluid-retaining properties of the hormone. Indeed, plasma levels of vasopressin tend to be raised in DOCA-salt hypertension. If the tubular sensitivity to vasopressin is concommitantly increased, as is the vascular sensitivity (Berecek et al., 1982), then these processes may lend themselves to the development of hypertension.

The present study shows that V_2 vasopressin receptors in basolateral membranes prepared from renal tubular epithelia in DOCA-salt treated rats are significantly elevated relative to tissue from non-DOCA treated animals. Animals treated with DOCA-salt and having a reduced renal mass (Group I) displayed a 56% increase in binding with respect to tissue from untreated animals (Group V) and a 71% increase compared to uninephrectomized animals receiving 1% NaCl (Group II). Those DOCA-salt hypertensive rats with intact kidneys (Group IV) displayed increases of 43% and 56%, respectively. The present finding that mineralocorticoids modulate the expression of vasopressin receptors in the kidney is consistent with the study of Rajerison et al. (1974). These authors observed that adrenalectomy resulted in a reduction of ^{3}H-lysine vasopressin binding in a renal medullary membrane preparation. A concomitant lowering of

vasopressin-stimulated adenylate cyclase activity was also noted. Replacement therapy with aldosterone restored the hormone binding capacity and adenylate cyclase activity to control levels. In the present study, vasopressin-stimulated cAMP production was commensurately increased in membranes prepared from the DOCA-salt hypertensive rats. Furthermore, the effective concentration which caused half-maximal stimulation (ED_{50}) was reduced. Thus, an increased sensitivity to vasopressin is suggested on the basis of adenylate cyclase activity, although no change in the affinity constant of the receptor was observed. Using microdissected renal tubules, Pettinger et al. (1986) have observed a similar increase in the sensitivity of adenylate cyclase in response to vasopressin in tubules obtained from DOCA-salt treated rats. This response was specific for vasopressin and occurred only in the segment of the cortical collecting tubule. The phenomenon was observed as early as five days post-treatment, a period which preceded the elevation of blood pressure.

The mechanism of vasopressin receptor up-regulation associated with DOCA-salt hypertension is not clear. It might have been expected that vasopressin binding would decrease in the face of elevated circulating levels of AVP, as was observed in Chapter 3. However, the magnitude of change in plasma vasopressin levels in the present study was only half of what occurred with dehydration. Clearly, factors other than homologous regulation must be invoked. As a steroid, DOCA probably acts by increasing the transcriptional rate for the vasopressin receptor protein. Increased expression of other tubular basolateral membrane proteins is also observed with

DOCA treatment. Garg et al. (1981) reported that Na-K-ATPase activity was markedly elevated in the cortical collecting duct of rabbits treated with DOCA. Similar results were observed by El Mernissi et al. (1983) and Mujais et al. (1985). Mineralocorticoids can affect the expression of receptors in other tissues as well. Wilson et al. (1986) observed a greater density of angiotensin II receptors in brain tissue. Angiotensin II receptors are also increased in vascular smooth muscle tissue in DOCA-salt hypertensive rats (Schiffrin et al., 1983). These findings correlate quite nicely with the increased dipsogenic and pressor responsiveness to Ang II in DOCA-salt treated animals. Not all receptors are modulated by mineralcorticoids, however, $Alpha_2$-adrenergic receptors were not significantly altered in membrane fractions prepared from DOCA-salt hypertensive and normotensive rats (Fukuda et al., 1983). These authors did observe differences however, in ^{3}H-yohimbine binding between spontaneously hypertensive rats and the their normotensive counterparts, Wistar-Kyoto rats.

How might an up-regulation of renal tubular vasopressin receptors contribute to the development of DOCA-salt hypertension? Vasopressin regulates fluid reabsorption in the distal nephron and also stimulates sodium reabsorption in at least two distinct sites: the medullary thick ascending limb (Hebert et al., 1981a) and in the cortical collecting tubule (Reif et al., 1986). A relationship between vasopressin-stimulated cAMP production and Na-K-ATPase activity, the enzyme involved in active transport of sodium, has been demonstrated by Tomita et al. (1987). As shown in the present study, the increased density of AVP receptors appears to render the tubule

more sensitive to vasopressin, reflected by the enhanced AVP-stimulated production of cAMP. Reif et al. (1986) have shown that chronic DOCA treatment enhances vasopressin-stimulated sodium reabsorption in the cortical collecting tubule. Treatment with DOCA also increases Na-K-ATPase activity in distal segments of the nephron (Garg et al., 1981). Thus, the increased concentration of plasma vasopressin noted in the present study coupled with an enhanced sensitivity to AVP in renal tubules may lead to an exaggerated retention of salt and fluids, resulting in an expanded blood volume. Extracellular volume expansion has been noted in at least some stage of the development of DOCA-salt hypertension (Villamil et al., 1982; Tajima et al., 1983).

Taken together, the present findings and the above studies suggest that the development of DOCA-salt hypertension may be caused, in part, by an increased tubular sensitivity to vasopressin. Increased activity of vasopressin-mediated processes would lend itself to a volume-expanded state, a condition necessary to prevent hypernatremia. Accordingly, the resultant blood pressure elevation could be viewed as a compensatory mechanism in response to an increase in cardiac output, secondary to hypervolemia and aimed at the restoration of fluid and electrolyte balance.

CHAPTER 5
EFFECTS OF MINERALOCORTICOIDS ON VASOPRESSIN RECEPTORS AND ADENYLATE CYCLASE-COUPLED RESPONSES IN LLC-PK_1 CELLS

Introduction

Although vasopressin can act on the kidney and affect renin secretion, glomerular filtration and tubular transport, a detailed understanding of how AVP or other hormones affect these processes at the cellular level has been difficult to ascertain. In large part, this is due to the complexity of the kidney and the veritable myriad of potential target tissues. One attempt to solve this problem has been to study the action of vasopressin on isolated glomeruli, vascular tissue or discretely dissected tubular segments. While somewhat more refined than an isolated whole kidney, this approach still ignores the heterogeneity of cell types within anatomically distinct regions. In order to circumvent these potential problems, one technique that has been applied to answer many of the questions concerning the cellular actions of vasopressin in the kidney is cell culture. The use of primary cultures and cell lines has been utilized successfully in a number of studies, ranging from transport physiology to hormonal regulation. Handler (1986) has recently written an elegant review on the use of kidney cells in culture and succinctly summarizes the wide-ranging studies amenable to these techniques.

The primary objective for using cell culture techniques in this study was to establish an in vitro system in which to evaluate the effects of mineralocorticoids on vasopressin binding, in order to

compare these results with those observed in vivo that are associated with mineralocorticoid-dependent hypertension. To this end, vasopressin binding was characterized in LLC-PK_1 cells (ATCC # CL 101), a continuous cell line of renal epithelial origin, established by Hull et al. (1976).

Materials and Methods

Chemicals

Tritiated 8-L-arginine [phenylalanine-3,4,5-^{3}H]-vasopressin (^{3}H-AVP) and tritiated 3',5'-cyclic adenosine monophosphate (^{3}H-cAMP) were purchased from New England Nuclear Corporation (Boston). Desoxycorticosterone acetate (DOCA), arginine and lysine vasopressin (AVP, LVP), oxytocin (oxy), angiotensin II (ang II), D-ala-met-enkephalinamide (DAME), cAMP and cAMP-dependent protein kinase were all purchased from Sigma Chemical Co. (St. Louis). Dulbecco's Modified Eagle's Medium and fetal bovine serum were obtained from GIBCO (Long Island, NY). All other reagents were obtained from Sigma, unless otherwise indicated.

Cell Culture

LLC-PK_1 cells (ATCC # CL 101) were routinely grown as monolayers in plastic 75 cm^2 Corning flasks using Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin [100 μg/ml], penicillin [100 units/ml], and $NaHCO_3$ [44 mM]. Cell cultures were maintained at 37°C in an atmosphere of 95% 0_2 and 5%CO_2. Dome formation typically appeared after several days of growth. Dome formation in epithelial cell cultures is acceptable presumptive evidence of the following processes that are required for its expression: functional plasma membrane

polarization, formation of occluding junctions and vectorial trans-epithelial active ion transport (Lever, 1985). At confluence, cells were subcultured as follows: the medium was removed and the cells washed with once with trypsin-EDTA solution (0.05% trypsin, 0.2% EDTA) for 10-15 minutes. The cells were centrifuged (500 x g, 5 min), resuspended in DMEM + 10% FBS and plated at a density of 2-2.5 x 10^4 cells/cm^2 in flasks as above for continued passage or in 35 mm plastic Falcon petri dishes and utilized upon achieving confluence.

Vasopressin Receptor Binding Assay

The vasopressin binding assay was carried out directly in the dish, on the monolayer. Twenty four hours prior to the experiment, the growth medium was removed and replaced with serum-free DMEM. At the time of the experiment, the medium was aspirated from the plates and the monolayer washed once with phosphate-buffered saline (PBS). PBS was composed of NaCl (8g/L), KCl (0.2g/L), Na_2HPO_4 (1.15g/L) and KH_2PO_4 (0.2g/L). Binding was initiated by the addition of 0.5 ml of the reaction buffer (PBS containing 0.8% bovine serum albumin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, pH 7.2) containing ^{3}H-AVP. Non-specific binding was determined in the presence of a 1000-fold excess of non-radioactive AVP. Studies were conducted in triplicate using ^{3}H-AVP at a concentration of 2.5 nM, for 30 minutes at 20°C, unless indicated otherwise and repeated at least once. For dissociation studies, the reaction medium was removed after 60 minutes and replaced with buffer containing 2.5 μM AVP. Saturation studies were performed with concentrations of ^{3}H-AVP ranging from 0.5-20 nM. Binding was terminated by rapidly aspirating the reaction mixture from the cells and washing twice with

ice-cold PBS. The cells were dissolved by the addition of 0.5 ml 0.1N NaOH, scraped free from the plate with a rubber policeman, transferred to a vial containing 10 ml of liquid scintillation fluid (Liquiscint), and counted for 5 minutes in an LKB liquid scintillation counter.

Stimulation of Adenylate Cyclase

Twenty four hours prior to the experiment, the growth medium was removed and replaced with serum-free medium. At the time of the experiment, the medium was aspirated and the cells washed once with PBS. The "control" buffer consisted of serum-free DMEM containing 11 μg/ml isobutyl-methyl xanthine (IBMX), a phospho-diesterase inhibitor and 0.1 mg/ml aprotinin, a serine protease inhibitor. The "stimulation" buffer consisted of the components described above, plus AVP in concentrations ranging from 1 nM to 1 μM. One ml of the control or stimulation buffer was added in triplicate to the plates and incubated at 37°C for 20 minutes. The reaction was terminated by rapidly aspirating the reaction mixtures, washing the monolayer once with PBS and adding 1 ml of boiling 1N HCl. The cells were scraped with a rubber policeman and the contents transferred into a 4 ml polypropylene tube with a Pasteur pipette. Samples were centrifuged at 3000 rpm for 5 minutes. The supernatants were removed and blown to dryness under a stream of air, before being stored at -20°C, for subsequent determination of cAMP formation. The pellets were saved for protein determination, according to the method of Lowry et al. (1951).

cAMP Determination

The cellular cAMP content was determined by the competitive protein binding assay as modified by Baker et al. (1986), as described in detail in Chapter 4.

DOCA Treatment of Cell Cultures

Desoxycorticosterone acetate (DOCA) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml. This stock concentration was initially diluted with PBS and subsequent dilutions, ranging from 1 pg/ml to 10 μg/ml were made in serum-free DMEM. The solutions were sterilized by passing them through 0.2 micron mesh Nalgene filters. Growth medium was aspirated off of the cells and replaced with DMEM containing DOCA. Cells were treated for 1-24 hours with DOCA concentrations ranging from 1 pg/ml to 1 μg/ml.

Results

Binding of ^{3}H-AVP to monolayers of LLC-PK$_1$ cells increased rapidly over time and attained equilibrium after 45 minutes. At this time, specific binding, represented ~75% of total ^{3}H-AVP bound. Upon equilibrium, binding was shown to be reversible by the removal of the radioligand and the addition of buffer containing unlabeled vasopressin. As with association, dissociation was rapid and reached levels equivalent to non-specific binding after 45 minutes (Figure 5-1). The specificity of the receptor was determined by competition of ^{3}H-AVP binding with a variety of related and unrelated peptides. Lysine^{8}-vasopressin and arginine^{8}-vasopressin were equally effective (IC_{50} = 2.1 nM) in terms of their ability of displacing the radioligand from the binding sites on the cells. On the other hand, oxytocin, angiotensin II and D-ala-met-enkephalinamide were ineffective even at

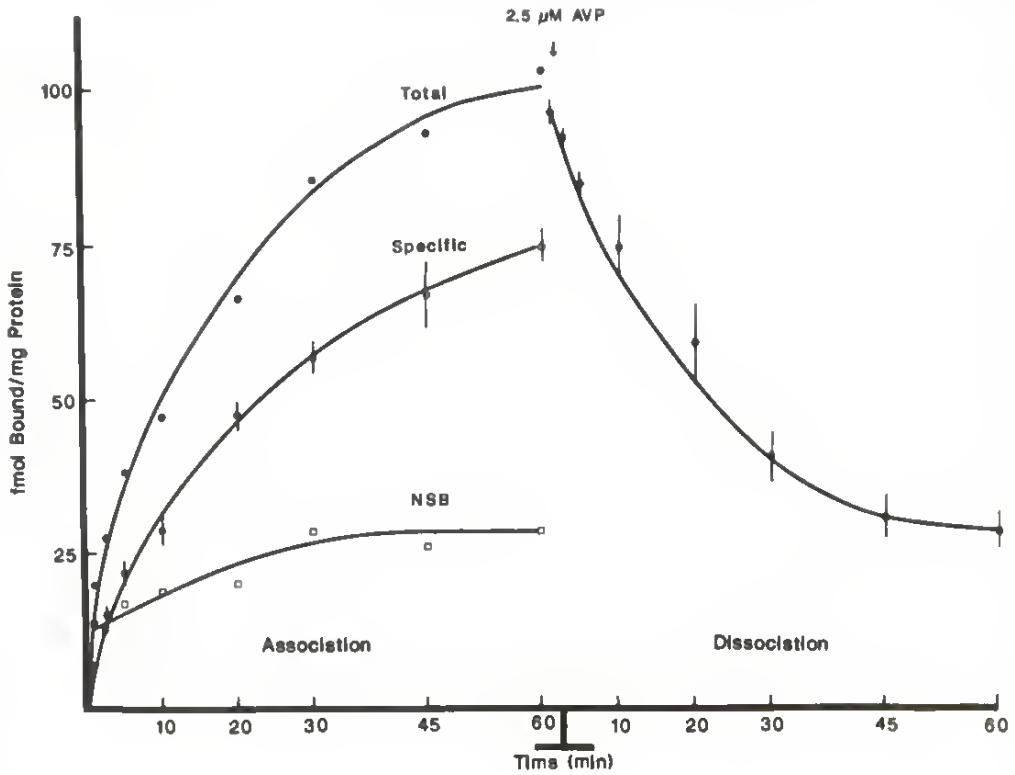

Figure 5-1. ^{3}H-vasopressin (^{3}H-AVP) binding in LLC-PK$_1$ cells as a function of time. Receptor binding protocol described in "Methods" section. Association: Total binding determined at 2.5 nM [^{3}H-AVP]. Non-specific binding (NSB) determined in the presence of 2.5 nM [^{3}H-AVP] + 2.5 μM unlabeled AVP. Total - NSB = Specific (75%). Dissociation: At equilibrium binding (1 hr), total ^{3}H-AVP binding is dissociated from receptor by the addition of 2.5 μM unlabeled AVP.

the highest concentration (10^{-6}M) in displacing ^{3}H-AVP (Figure 5-2). Using increasing concentrations of ^{3}H-AVP, binding sites on the surface of the cells appear to be saturable (Figure 5-3). Scatchard analysis of the saturation isotherm data reveals a single class of high affinity binding sites (K_D = 2.98 nM). This is in good agreement with the value of 2.1 mM observed for the IC_{50} for vasopressin. The maximal binding capacity (B_{max}) was calculated to be 155 fmoles/mg protein (Figure 5-3, insert). Vasopressin stimulated cAMP accumulation in a dose-dependent manner with LLC-PK_1 cells (Figure 5-4) which was abolished by pre-treatment with V_2 antagonists. Treatment of the cell cultures with DOCA, either over varying periods of time or with varying concentrations, failed to alter the binding of ^{3}H-AVP (Figure 5-5). While binding was significantly reduced at 1 μg/ml DOCA, this effect can be attributed to the "toxic" effect of the mineralocorticoid, as cell viability was adversely affected. Basal and vasopressin-stimulated cyclic AMP formation in LLC-PK_1 cells were not significantly affected by DOCA treatment (Figure 5-6).

Discussion

The structural and functional heterogeneity of the kidney is not particularly amenable to the study of hormonal action at the cellular level. One attempt to resolve this dilemma has been the use of tissue cultures of specific cellular elements in the kidney. These techniques have facilitated our understanding of vasopressin action at targets as diverse as glomerular mesangial cells (Bonventre et al., 1986) and tubular epithelial cells (Tang and Weinberg, 1986). In the present study, the interaction of vasopressin with its receptor was

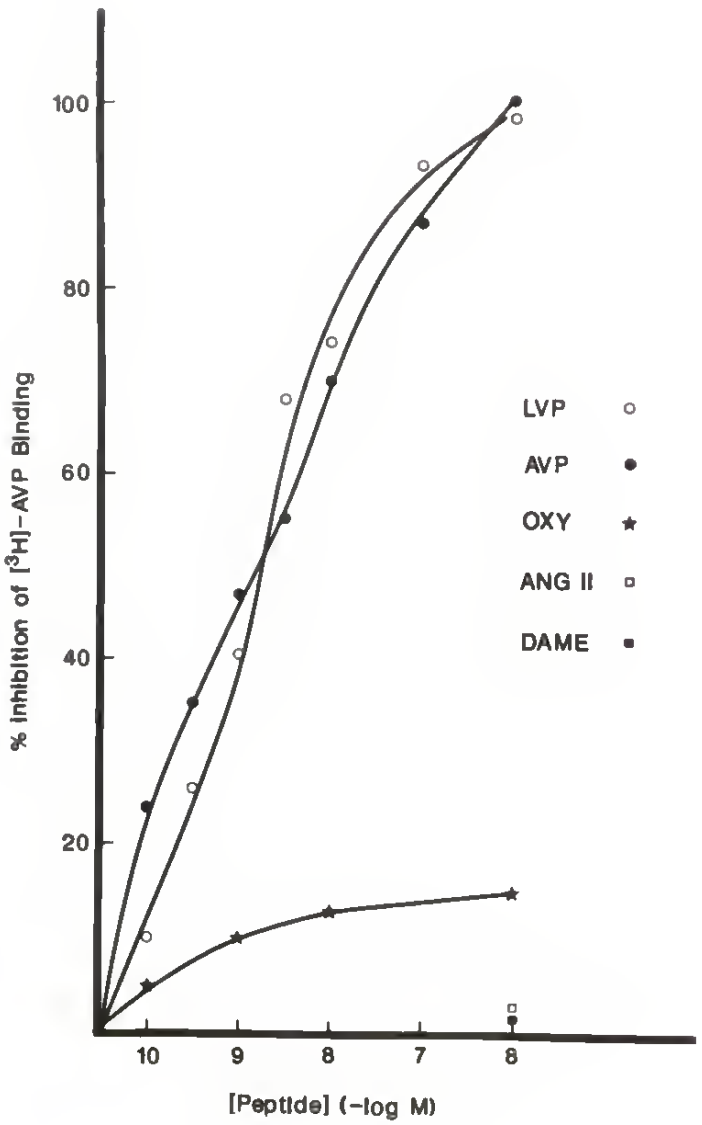

Figure 5-2. Specificity of vasopressin receptor in LLC-PK_1 cells. Competition of ^{3}H-vasopressin (^{3}H-AVP) binding with vasopressin analogues and unrelated peptides. Receptor binding protocol described in "Methods" section. Abbreviations are as in Figure 2-4.

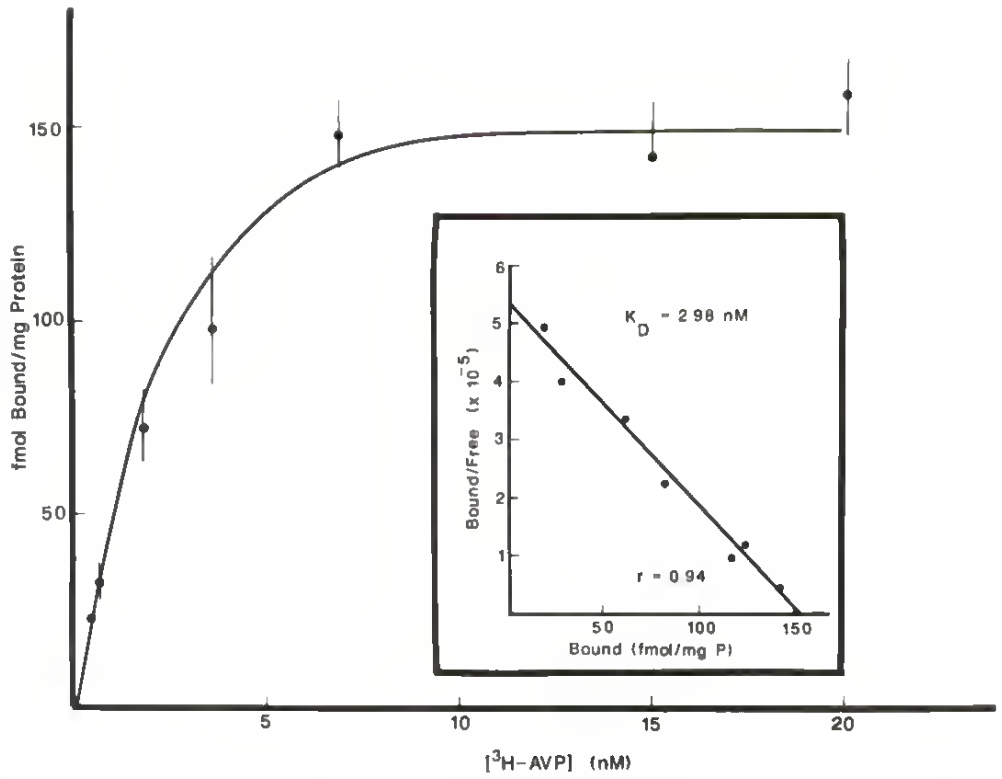

Figure 5-3. Saturation isotherm and Scatchard plot (insert) of ^{3}H-vasopressin (^{3}H-AVP) binding in LLC-PK$_1$ cells. Concentration of ^{3}H-AVP ranged from 500 pM to 20,000 pM. Specific binding was determined using a thousand-fold excess of unlabeled AVP. Details of the binding procedure are described in the "Methods" section.

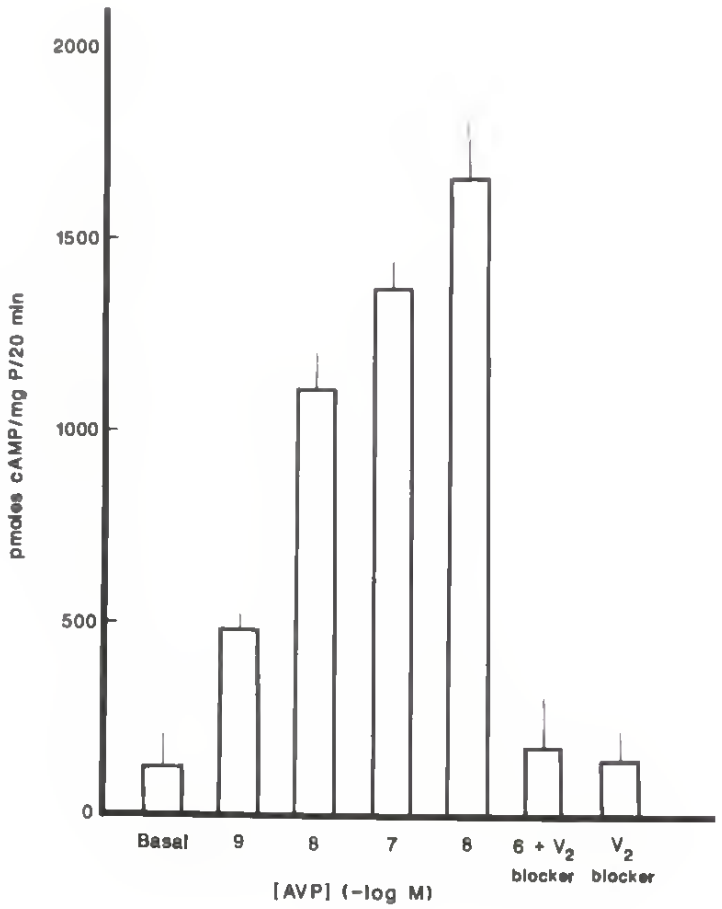

Figure 5-4. Basal and vasopressin-stimulated cyclic AMP formation in LLC-PK_1 cells. Procedures for the stimulation and determination of cyclic AMP are described in the "Methods" section. AVP, arginine vasopressin; cAMP, adenosine 3'5'-cyclic monophosphate; V_2 blocker, $d(CH_2)_5$D-Ile^2-Ile^4-AVP.

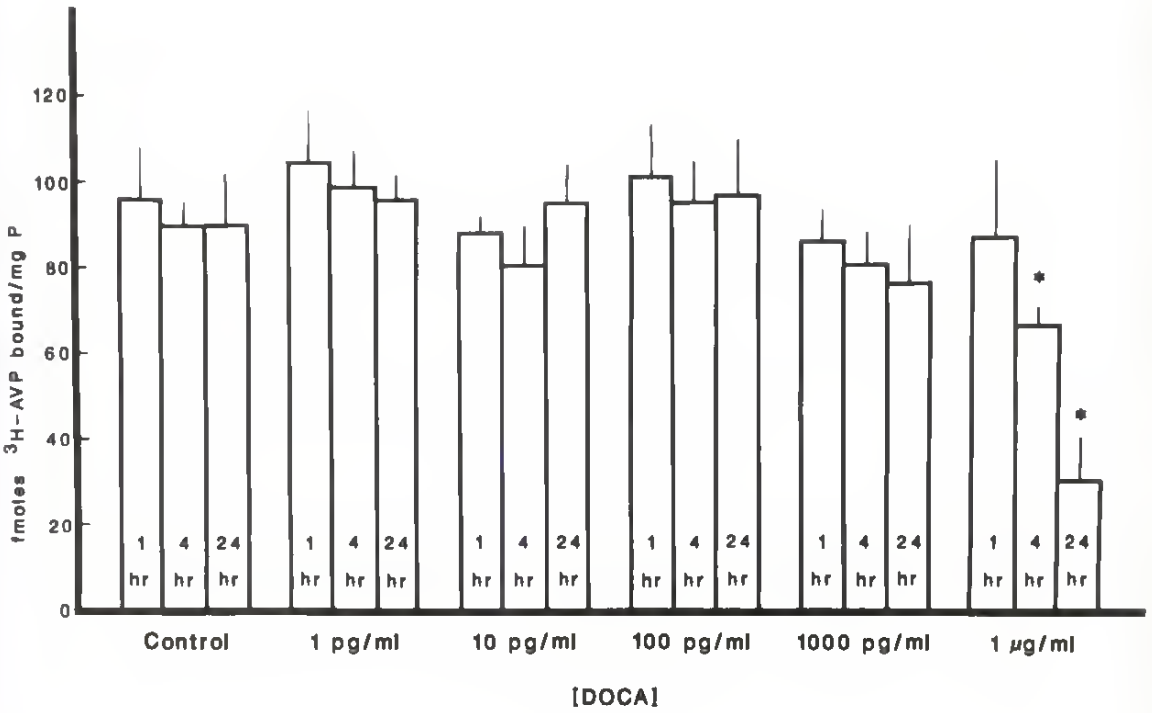

Figure 5-5. Effect of DOCA on ^{3}H-vasopressin (^{3}H-AVP) binding in LLC-PK$_1$ cells. Cells were treated for 1-24 hours with DOCA ranging in concentration from 1 pg/ml to 1 µg/ml. Binding was assessed at 3.0 nM [^{3}H-AVP], with details provided in the "Methods" section. DOCA, desoxycorticosterone acetate. *p.05 vs. control.

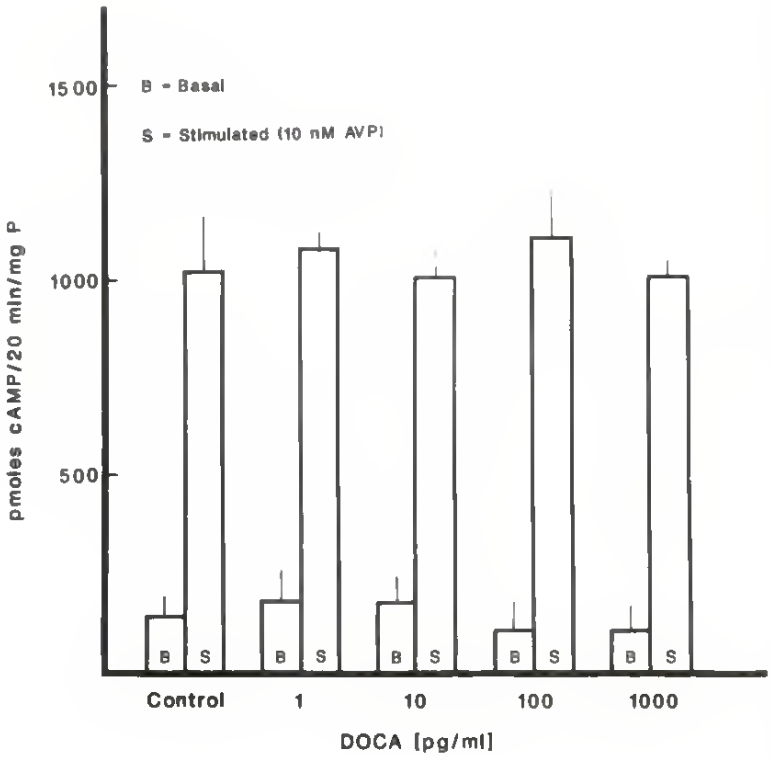

Figure 5-6. Effect of DOCA on basal and vasopressin-stimulated cAMP production in LLC-PK_1 cells. Basal cAMP was measured in the absence of AVP. Stimulated values were assessed in the presence of 10 nM AVP. Details of procedures are found in the "Methods" section. DOCA, desoxycorticosterone acetate; AVP, arginine vasopressin; cAMP, adenosine 3'5'-cyclic monophosphate.

examined in a homogeneous population of tubular epithelial cells and the influence of mineralocorticoids on AVP binding and adenylate cyclase activity was determined.

Binding of ^{3}H-arginine vasopressin to LLC-PK$_1$ cells was rapid, reversible and specific. The receptor displayed a high affinity (2-3 nM) for arginine vasopressin and the closely related moiety, lysine vasopressin, but for no other peptide, including oxytocin. This finding differs somewhat from that reported by Roy and Ausiello (1981a) who found that the IC_{50} for AVP was more than an order of magnitude greater than that for LVP. This may be due to differences in the radioligands used between the two studies. Roy and Ausiello utilized a tritiated lysine vasopressin compound with a specific activity of 8.5 Ci/mmol, whereas the tritiated vasopressin analogue used in the present study contained arginine in the number 8 position of the peptide and had a specific activity eight times greater (70 Ci/mmol). Variability in the phenotypic expression of the cells from passage to passage must also be considered when interpreting any result. Scatchard analysis performed on the saturation data in the present study revealed a single class of binding sites with an apparent B_{max} of 155 fmol/mg protein. This finding too differs from those of Roy and Ausiello (1981a). They reported a nonlinear plot, indicative of heterogeneity in the population of binding sites. Comparison of the maximal binding capacities for the high affinity sites in each of the studies is difficult, as Roy and Ausiello (1981a) expressed their data on the basis of cell number, rather than per mg of protein. However, the B_{max} of 155 fmols/mg protein calculated in

the present study is in close agreement with the value of 166 fmoles/mg protein, as determined by Jans et al. (1987).

Roy and Ausiello (1981b) found that cells suspended in hypertonic saline or treated with EDTA exhibited a homogeneous population of binding sites. They proposed a hypothesis for vasopressin-receptor interactions involving a hormone-induced alteration in binding affinity or so-called "receptor transition". In other words, rather than postulating two independent binding sites, as a nonlinear Scatchard plot might suggest, they believe in an interconversion of receptor states of different affinity. Why a similar situation was not observed in the present study is unclear.

The binding sites characterized in the present study apparently represent vasopressin receptors, as vasopressin was shown to stimulate cAMP production in a dose-dependent manner. Agonist-induced stimulation resulted in a sixteen-fold increase over basal levels. This effect was completely abolished by pretreatment of the cells with a V_2 antagonist. The values for basal and AVP-stimulated cAMP levels are in good agreement with those reported by Lester et al. (1985) and Skorecki et al. (1987).

The synthetic mineralocorticoid DOCA, had no demonstrable effect on either vasopressin binding or cAMP production in LLC-PK_1 cells in this study. This contrasts with the observations noted in vivo, as described in Chapter 4. In that study, a significant increase was found in the vasopressin-stimulated adenylate cyclase activity and number of vasopressin binding sites on tubular epithelial membranes prepared from the kidneys of rats chronically treated with DOCA and NaCl.

It may be appropriate to consider several possibilities to account for the lack of mineralocorticoid effect noted in the LLC-PK_1 cells. The most obvious explanation is that this particular cell line does not possess receptors for mineralocorticoids, whereas cytosolic and nuclear binding sites for mineralocorticoids have been reported for A_6 cells (Handler et al., 1981) and MDCK cells (Luden et al., 1978). No such reports have appeared in the literature for LLC-PK_1 epithelial cells. This is consistent with the observation that LLC-PK_1 cells, unlike the A_6 and MDCK epithelial cell lines, are relatively refractory to the effects of aldosterone, at least with respect to transepithelial Na^+ transport (Meier and Insel, 1985).

Perhaps the most pertinent evidence concerning the lack of effect of mineralocorticoids in LLC-PK_1 cells comes from a study by Roy et al. (1980). These investigators examined the regulation of vasopressin receptors in LLC-PK_{1L} cells, a subline of the parent LLC-PK_1 strain. These cells are characterized by possessing only 5% as many vasopressin receptors as the parent cell line. They reported that fetal bovine serum or insulin could markedly stimulate the expression of receptors in these cells. In contrast, thyroid hormone, dexamethasone and aldosterone were ineffective in evoking a change in receptor number.

Alternatively, methodological considerations must not be overlooked. DOCA was only soluble in organic solvents such as absolute ethanol or DMSO. Dilution of the DOCA-containing solvents with aqueous buffers often resulted in the precipitation of the steroid. Thus, it is impossible to say with any degree of certainty exactly how much DOCA was applied to the cell cultures. On the

other hand, at high doses (>1 μg/ml), viability of the cells became a significant factor. One study reported that ^{3}H-DOCA receptor binding was inhibited by a broad class of protease inhibitors (Baker and Fanestil, 1977). Whether mineralocorticoids exert any physiological effects in LLC-PK$_1$ cells remains in question, but it is entirely possible that the use of protease inhibitors in the present study may have antagonized the effect of the steroid.

Thus, vasopressin receptors are expressed in vivo on renal tubular epithelial membranes and in vitro on renal epithelial cells. Both receptors are coupled to adenylate cyclase. In addition, both receptors are susceptible to regulation by vasopressin (Chapter 3 and Lester et al., 1985). It appears however, that the receptors respond differentially to the effects of mineralocorticoids. The question of whether mineralocorticoids directly affect the expression of vasopressin receptors at the cellular level remains unanswered using LLC-PK$_1$ cells as a model. The question may be more suitably addressed using a primary culture of epithelial cells prepared from rat collecting tubules.

CHAPTER 6
INTRACELLULAR SECOND MESSENGERS IN LLC-PK_1 CELLS: A DUAL TRANSMEMBRANE SIGNALING SYSTEM

Introduction

It is now well-documented that vasopressin receptors are integral components in the plasma membrane of LLC-PK_1 cells, a continous cell line of renal epithelial origin (Ausiello et al., 1987). Vasopressin interacts with these receptors to initiate the sequence of intracellular events that ultimately lead to the transepithelial transport of fluid and solutes, in a manner not unlike that seen in the distal collecting segments of the nephron. As in the kidney, vasopressin receptors in LLC-PK_1 cells display characteristics of the V_2 subtype and are coupled to adenylate cyclase, resulting in a hormone-specific stimulation of cAMP accumulation (Goldring et al., 1978; Roy and Ausiello, 1981a). Recently, it was reported that vasopressin stimulated transient increases in cytosolic calcium in LLC-PK_1 cells (Tang and Weinberg, 1986). This action is not unlike the vasopressin-stimulated calcium fluxes noted in hepatocytes (Michell et al., 1979) and smooth muscle cells (Aiyar et al., 1986). In these tissues however, vasopressin exerts its action through V_1 receptors, which are coupled to phospholipase C and the hydrolysis of phosphatidyl inositol (PI). This leads to the generation of inositol phosphates (IP) and diacylglycerol (DG). Inositol triphosphate (IP_3) is thought to be involved in the mobilization of calcium from intracellular organelles such as the endoplasmic reticulum whereas DG

has been shown to be an endogenous activator of protein kinase C (Berridge, 1987), as illustrated in Figure 6-1.

Roy and Ausiello (1981b) have demonstrated that the vasopressin receptor-effector system in LLC-PK_1 cells can be modulated by altering the osmolality of the medium to which the cells are exposed. Variation in osmotic conditions is analogous to the situation in the kidney where segments of the tubule are exposed to a continous corticomedullary osmotic gradient. Thus, the epithelial cells and the receptors situated on the plasma membranes of their surfaces are exposed to conditions ranging from isotonicity to hypertonicity. Roy and Ausiello (1981b) reported that incubation in hypertonic NaCl will increase the binding of vasopressin and the coupling between the receptor and adenylate cyclase, leading to elevated levels of cAMP.

Thus, while it is well known that vasopressin stimulates the production of cAMP in LLC-PK_1 cells, the following study was designed to determine if vasopressin stimulates the hydrolysis of phosphatidyl inositol, resulting in the formation of inositol phosphates. Furthermore, the influence of increasing the osmolality of the incubation medium on vasopressin-stimulated PI hydrolysis was also examined.

Materials and Methods

Chemicals

Peptides (arginine vasopressin, lysine vasopressin, angiotensin II) and the cholinergic agonist carbachol were purchased from Sigma (St. Louis). Vasopressin antagonists were a gift from Dr. M. Manning of the Medical College of Ohio. ^{3}H-myo-inositol and OCS liquid scintillation fluid were obtained from Amersham (Arlington Hts., IL).

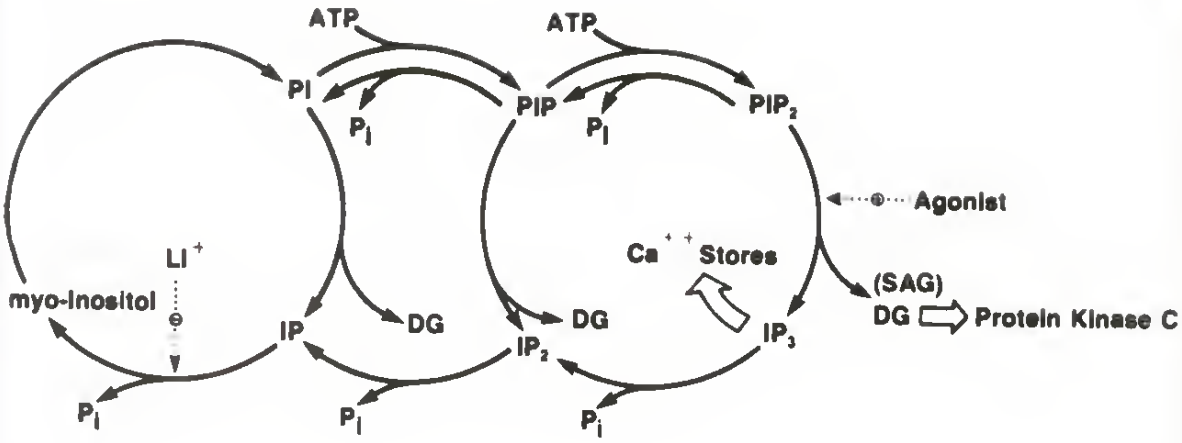

Figure 6-1. Phosphoinositide cycle. Myo-inositol is incorporated in phosphoinositides (PI, PIP, PIP_2). Agonist-occupied receptors stimulate phospholipase C, which acts to cleave PIP_2 and IP_3 and DG. IP_3 acts to mobilize intracellular stores of calcium, then IP_3 is metabolically converted to IP_2 and IP under the action of the appropriate phosphatases. DG activates a calcium-phospholipid-dependent protein kinase C, which can phosphorylate a number of cellular proteins. ATP, adenosine triphosphate; PI, phosphatidyl inositol; PIP, phosphatidyl inositol-4-phosphate; PIP_2, phosphatidyl inositol-4,5-phosphate; DG, diacylglycerol; IP_3, inositol 1,4,5-phosphate; IP_2, inositol 4-phosphate; IP, inositol 1-phosphate; P_i, inorganic phosphate.

Dowex was obtained from Biorad Laboratories (New York). Liquiscint liquid scintillation fluid was purchased from National Diagnostics (New Jersey). Dulbecco's Modified Eagle's Medium and fetal bovine serum were obtained from GIBCO (New York). Other reagents were obtained from Fisher Scientific (Orlando) unless indicated otherwise.

Culturing and Preparation of LLC-PK_1 Cells

LLC-PK_1 cells (ATCC # CL 101) were routinely grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. Cells were plated at an initial density of 2-2.5 x 10^4 cells/cm^2 in plastic 75 cm^2 flasks and on 35 mm plates. The cultures were maintained at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. Cells in the 35 mm plates were utilized upon achieving confluence whereas the monolayers in the flasks were subcultured according to the procedure described in Chapter Five. The cells to be utilized for the experiments were washed twice with buffer I, a regular Krebs-Ringer Bicarbonate (KRB) buffer, which contained 118 mM NaCl, 4.7 mM KCl, 0.75 mM $CaCl_2$, 1.18 mM KH_2PO_4, 24.8 mM $NaHCO_3$ and 10 mM glucose and had an osmolality of 300 mOsm/kg H_2O. The cells from each plate were scraped with a rubber policeman, transferred to a tube containing KRB buffer and centrifuged at 500 x g for 5 minutes. The cells were then resuspended in the appropriate buffer. In some experiments, buffers of different osmolality than that of buffer I were used. Buffer II (600 mOsm/kg H_2O) was prepared by the addition of urea (300 mOsm/kg H_2O) to buffer I (which had NaCl as its major component). Buffers III (900 mOsm/kg H_2O) and IV (1200 mOsm/kg H_2O) were prepared by the addition of equiosmolar amounts of NaCl and urea to

buffer II. In this way, the buffers simulated the osmolality and composition of the renal medullary interstitium.

Incorporation of ^{3}H-inositol into Membrane Phosphoinositides and Release of ^{3}H-inositol Phosphates

The method for measurement of phosphoinositide (PI) hydrolysis in LLC-PK$_1$ cells was similar to that described by Gonzales and Crews (1984) for brain slices. LLC-PK$_1$ cells were incubated with 0.1 μM ^{3}H-inositol (Specific activity = 16.0 Ci/mmol) in the appropriate buffer (I to IV) for 3 hours at 37°C in a shaking water bath. The cells were then washed with buffer of the same osmolality (except that it did not contain ^{3}H-inositol) and divided into several aliquots. In each aliquot, more of the same buffer but containing 20 mM LiCl was added. LiCl inhibits dephosphorylation of inositol phosphates (IP), and has been used to amplify hormone-dependent PI responses in numerous tissues including LLC-PK$_1$ cells (Berridge, 1987; Kinoshita et al., 1987).

To determine the hormone-stimulated release of ^{3}H-IP, the reaction was started by adding vasopressin or other agents and incubating the samples at 37°C in a shaking water bath. During incubation the buffers were kept saturated with O_2:CO_2 (95:5) mixture. After one hour, the reaction was terminated by the addition of chloroform:methanol (1:2 v/v) mixture. The organic and aqueous layers were separated by centrifugation.

Quantitation of Inositol Phosphates (IP) Released

In order to eliminate the variation due to differences in number of cells or ^{3}H-inositol incorporation in each sample, values are expressed as ^{3}H-inositol phosphates released as a percentage of the total amount of ^{3}H-inositol incorporated in each sample. To do this,

radioactivity was determined in the organic layer (which contained unhydrolyzed ^{3}H-phosphoinositides) and in the aqueous layer (which contained ^{3}H-inositol phosphates). An aliquot of the organic layer was placed in a scintillation vial and the chloroform was allowed to evaporate in a fume hood. The radioactivity in the vial was determined by the addition of OCS scintillation fluid and counted in a Beckman (LS 7000) scintillation counter. The aliquots of aqueous layers were mixed with Dowex (50% v/v, 100-200 mesh, formate form) and poured into polypropylene columns. After the liquid was drained, the Dowex was washed with 5 mM (unlabeled) inositol to remove free ^{3}H-inositol. Total ^{3}H-inositol phosphates were eluted with 0.1 M formic acid/1.0 M ammonium formate. The radioactivity of the eluted ^{3}H-inositol phosphates in each sample was measured by adding Liquiscint and counted in a Beckman (LS 7000) scintillation counter. The release of ^{3}H-inositol phosphates (IP) were calculated as follows:

$$[^{3}\text{H}]\text{-IPs RELEASED (\% Total Incorp.)} = \frac{\text{dpm of IP in aqueous layer}}{\text{dpm of IP in aq. layer + dpm of PI in org. layer}}$$

Statistical Analysis

The data on the release of IP with different drugs or in different buffers were analyzed by one-way ANOVA (analysis of variance). The individual contrasts between treatment pairs were made by Duncan's method. When only two groups were used, the data were analyzed by the Student t-test. Differences with $P < 0.05$ were considered significantly different from each other.

Results

After incorporation of ^{3}H-inositol into phosphatidyl inositol of the membranes of LLC-PK$_1$ cells for 3 hours, the vasopressin-

stimulated release of inositol phosphates was determined. The data in Figure 6-2 indicate that vasopressin-stimulated IP release increased with time at least up to 120 minutes. Sixty minutes was chosen as the incubation period for measurement of IP release from radiolabeled PI in LLC-PK_1 cells.

Stimulation of PI hydrolysis in LLC-PK_1 cells by vasopressin proceeded in a dose-dependent manner, as seen in Figure 6-3. At at concentration of 10^{-8} M, there was a > 100% increase in PI hydrolysis in these cells. The effect was dose-dependent up to 10^{-5} M vasopressin in the incubation medium.

The specificity of the agonist-stimulated PI hydrolysis is illustrated in Figure 6-4. Both arginine vasopressin and lysine vasopressin in equimolar concentrations (10^{-7} M) significantly stimulated PI hydrolysis when compared to the incubation buffer ($p<.05$). Carbachol, a cholinergic agonist that has been reported to stimulate PI hydrolysis in kidney slices (Garg et al., 1986), exerted no significant effect in LLC-PK_1 cells at concentrations up to 1 mM. Angiotensin II, an octapeptide which is structurally different from vasopressin but shares some physiological and biochemical properties (vasoconstrictor, stimulates PI hydrolysis in vascular smooth muscle cells), failed to stimulate PI hydrolysis in LLC-PK_1 cells at concentrations up to I0 μM. An attempt was made to deduce the nature of the vasopressin receptor mediating the PI response seen in this cell system. As can be seen in Figure 6-5, the V_1 antagonist significantly reduced the amount of vasopressin-stimulated PI hydrolysis ($p<.05$). The V_2 receptor blocker also significantly lowered the amount of vasopressin-stimulated release of PI ($p<.05$). Both the

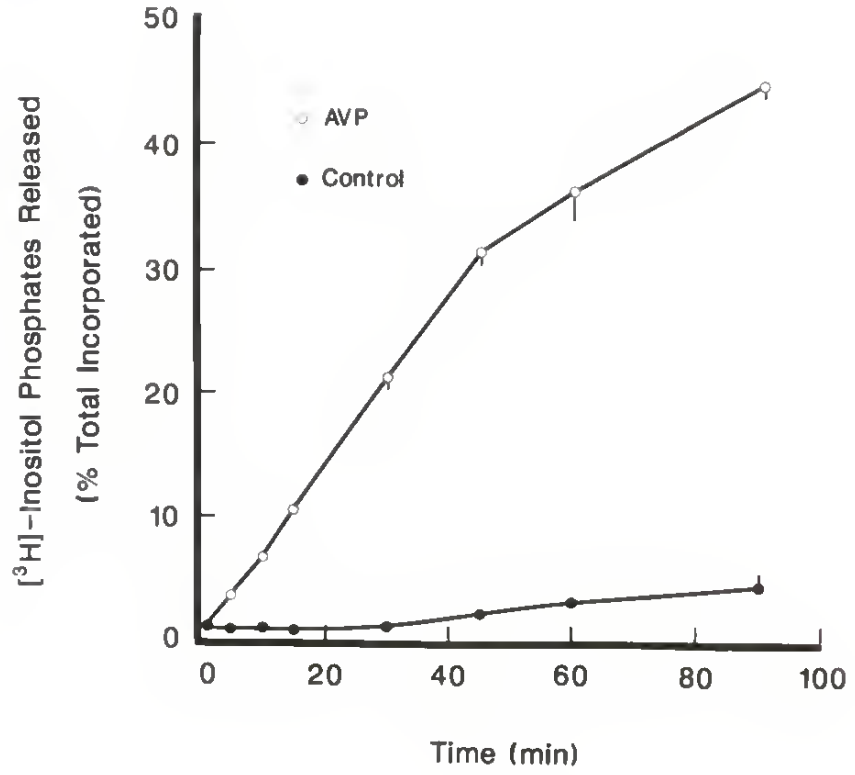

Figure 6-2. Time dependence of ^{3}H-inositol phosphate release in LLC-PK$_1$ cells. Control represents Krebs-Ringers buffer. Vasopressin (AVP)-stimulated release was conducted at a concentration of 10 μM AVP. Results are expressed as ^{3}H-inositol incorporated into the phosphatidyl inositol pool. Details of the procedure are found in the "Methods" section.

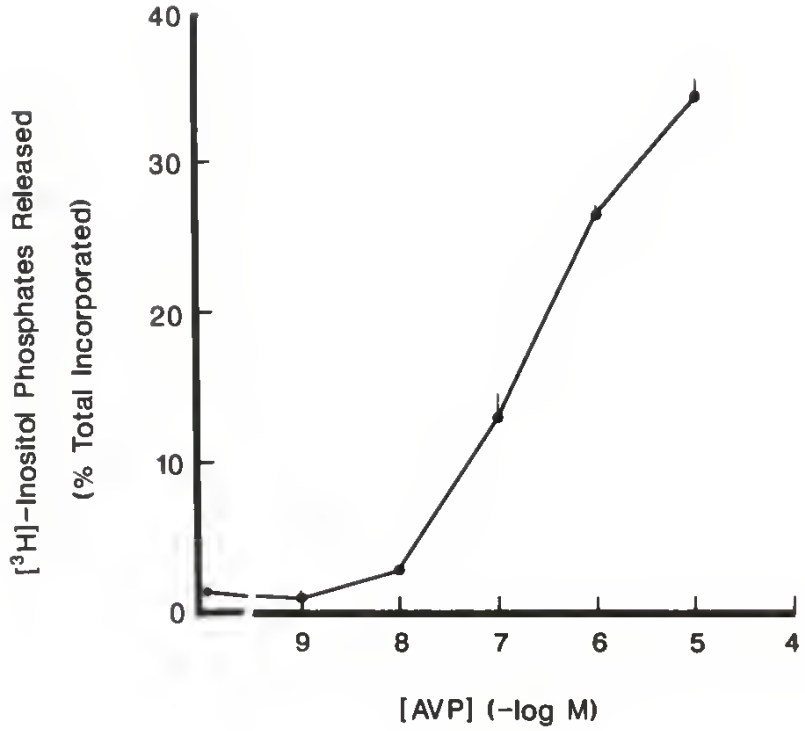

Figure 6-3. Dose-dependent stimulation of ^{3}H-inositol phosphate release in LLC-PK$_1$ cells. Vasopressin (AVP), in concentrations ranging from 1 nM to 10 μM, were used to stimulate the release of ^{3}H-inositol phosphate. Results are expressed as in Figure 6-2. Details of the procedure are found in the "Methods" section.

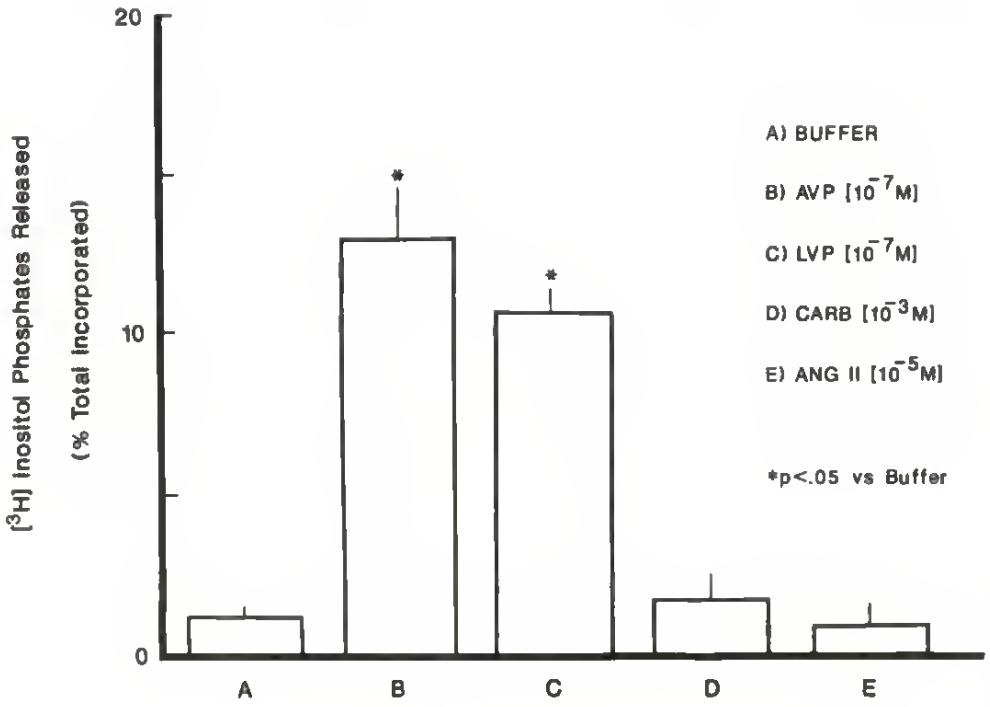

Figure 6-4. Specificity of agonist-stimulated ^{3}H-inositol phosphate release in LLC-PK$_1$ cells. Peptides and the muscarinic agonist carbachol were used, in the concentrations indicated to stimulate ^{3}H-inositol phosphate release. Results are expressed as in Figure 6-2. Details of the procedure are found in the "Methods" section. AVP, arginine vasopressin; LVP, lysine vasopressin; CARB, carbachol; ANG II, angiotensin II.

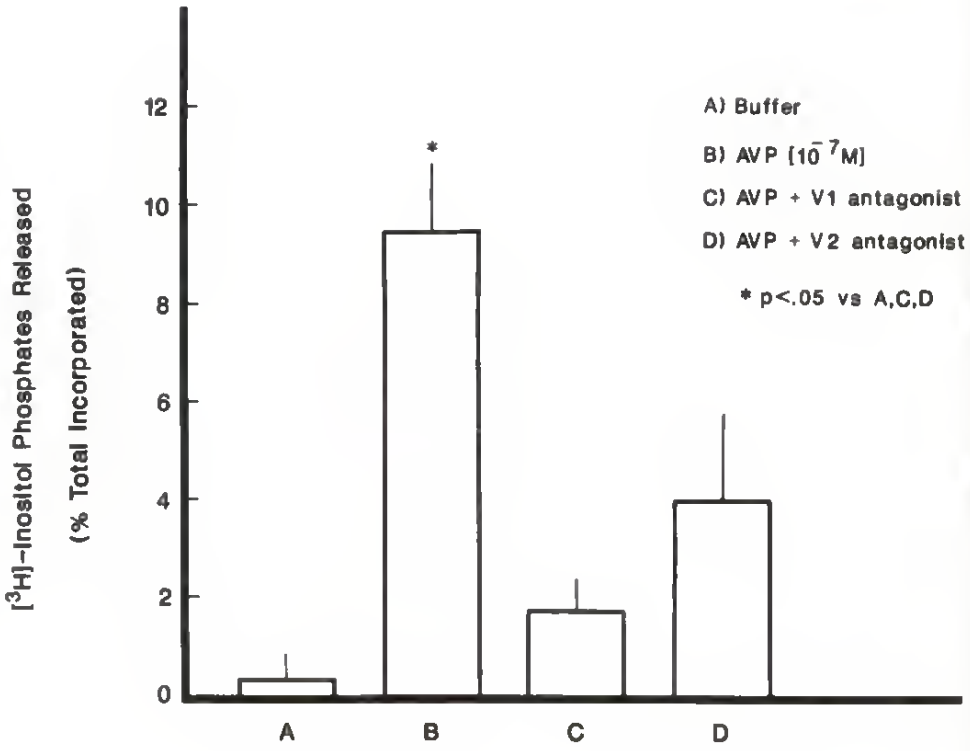

Figure 6-5. Antagonism of vasopressin-stimulated ^{3}H-inositol phosphate release in LLC-PK$_1$ cells. Vasopressin at a concentration of 100 nM, stimulated the release of ^{3}H-inositol phosphate. This effect was totally abolished by pretreatment with equimolar amounts of the V_1 antagonist and attenuated by the V_2 antagonist. Results are expressed as in Figure 6-2. Details of the procedure are found in the "Methods" section. Abbreviations for the V_1 and V_2 antagonists are as in Figure 2-4. AVP, arginine vasopressin.

V_1 and V_2 blockers by themselves caused a small but statistically non-significant stimulation of PI hydrolysis (data not shown).

The effect of increasing the osmolality of the incubation medium was assessed on vasopressin-stimulated PI hydrolysis. These results are presented in Figure 6-6. Raising the osmolality to 600 mosmol/kg H_20 had no significant effect on AVP-evoked PI release. However, when the osmolality was increased further, to 900 and 1200 mosmol/kg H_20, vasopressin-stimulated PI hydrolysis was significantly reduced with respect to the isoosmotic buffer ($p<.05$). Altering the osmotic environment had no significant effect on the buffer-evoked release of PI.

Discussion

The vast preponderance of evidence suggests that vasopressin causes the transepithelial transport of fluid and solutes in mammalian renal tubules and amphibian urinary bladder by stimulating adenylate cyclase which leads to the activation of cAMP-dependent protein kinases. Vasopressin-stimulated cAMP production has also been demonstrated in LLC-PK_1 cells, epithelial cells of renal epithelial origin (Goldring et al., 1978; Chapter 5). The experiments conducted in the present study represent the first documentation of vasopressin-stimulated hydrolysis of phosphatidyl inositol in cells of renal epithelial origin.

The effect of AVP-stimulated PI hydrolysis was time- and dose-dependent, with the minimum effective dose being 10^{-8} M. While this concentration of vasopressin is generally higher than that required to stimulate adenylate cyclase activity in these cells (Goldring et al., 1978; Ausiello et al., 1987), the value is identical to that required to

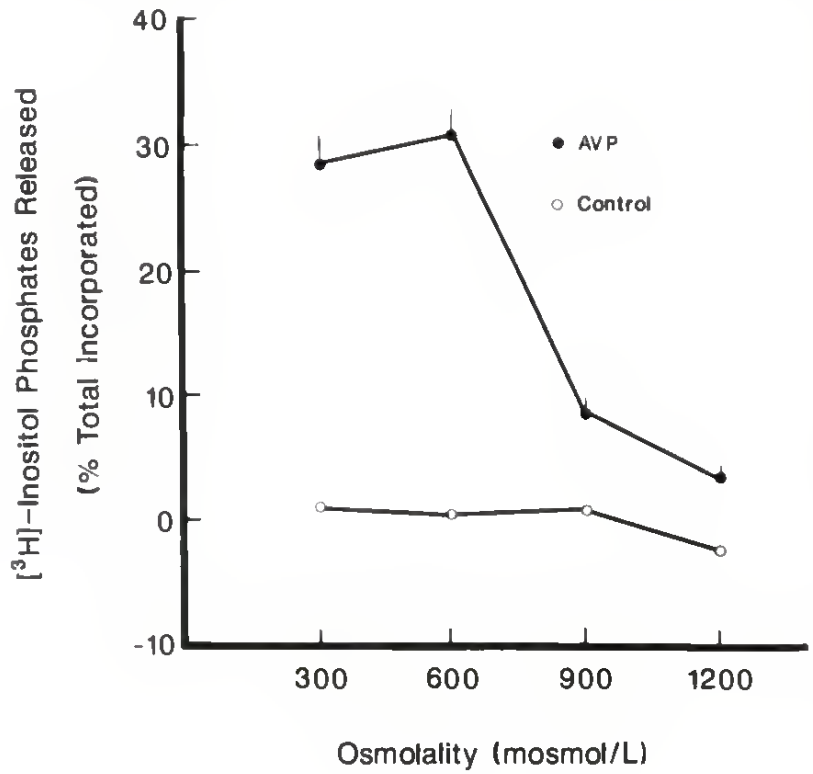

Figure 6-6. The effect of osmolality on ^{3}H-inositol phosphate release in LLC-PK$_1$ cells. Osmolality was increased from normal iso-osmotic conditions by the addition of NaCl and urea. This mimics the corticomedullary osmotic gradient as seen in the kidney. Control represents Krebs-Ringers buffer. Vasopressin (AVP), at a concentration of 1 μM, was used to stimulate release. Results are expressed as in Figure 6-2. Details of the procedure are found in the "Methods" section.

elicit an increase in cytosolic calcium in these cells (Tang and Weinberg, 1986). Why the concentrations of vasopressin necessary to stimulate these second messenger systems are higher than circulating levels of vasopressin in vivo is unclear. However, it should be noted that in certain tissues (kidney, urinary bladder), activation of only a small fraction of hormone receptors is necessary to produce maximal physiological responses.

Only arginine- and lysine-vasopressin were effective in stimulating PI hydrolysis in LLC-PK_1 cells. The muscarinic agonist carbachol and angiotensin II were without effect in this cell system. This differs from the findings of Garg et al. (1986 and unpublished observations) in which the cholinergic agent and peptide were shown to stimulate PI hydrolysis in kidney slices. This suggests that the stimulation of PI hydrolysis in LLC-PK_1 cells by vasopressin is hormone-specific.

The nature of the receptors which mediate the vasopressin-induced release of inositol phosphates from the membranes of LLC-PK_1 cells remains to be clarified. Two major subtypes of vasopressin receptors have been characterized, based on the effector system to which they are coupled. V_1 receptors, which mediate the glycogenolytic action in hepatocytes and the vasoconstrictor action in smooth muscle cells, are coupled to the hydrolysis of phosphatidyl inositol and the generation of inositol phosphates and diacylglycerol. V_2 receptors on the other hand, mediate the antidiuretic action of the hormone and are linked to adenylate cyclase and the production of cAMP (Michell et al., 1979). Based on these criteria, it would appear that the vasopressin-induced release of inositol phosphates in

LLC-PK_1 cells as seen in the present study, is mediated through V_1-type vasopressin receptors. The use of selective V_1 and V_2 antagonists proved somewhat equivocal, however. A series of these compounds have been developed which antagonize the vasoconstrictive and antidiuretic actions of the hormone, respectively (Sawyer et al., 1981; Manning and Sawyer, 1986). In the present study, vasopressin-stimulated PI hydrolysis was completely abolished by pretreatment with the selective V_1 antagonist $d(CH_2)_5$-Tyr(Me)-AVP. The finding that the V_2 antagonist $d(CH_2)$-D-Ile^2-Ile^4-AVP partially blocked the vasopressin-induced release of inositol phosphates was rather surprising. Several explanations may be considered when interpreting these data. This first deals with the relative potency displayed by $d(CH_2)$-D-Ile^2-Ile^4-AVP. This compound displays only a forty-fold greater anti-antidiuretic activity than anti-vasopressor activity, compared to $d(CH_2)_5$-Tyr(Me)-AVP, which is the most potent anti-vasopressor available and displays little or no antidiuretic-blocking activity (Manning and Sawyer, 1986). Alternatively, the vasopressin receptor present in LLC-PK_1 may display different physical characteristics from vasopressin receptors for which these analogues were designed. Kinter et al. (1988) have noted several instances where the selectivity of V_2 antagonists is greatly reduced.

Another factor that should be considered is that V_2 antagonists also inhibit vasopressin-stimulated cAMP production. This points out the possible interaction between the two intracellular signaling systems. Berridge (1987) describes several examples of interactions between the PI and adenylate cyclase pathways. Friedlander and Amiel (1987) reported that activators of protein kinase C inhibit

vasopressin-stimulated cAMP formation in MDCK cells. This action may occur by covalent modification of the vasopressin receptor or the adenylate cyclase enzyme. This latter concept is strengthened by the finding of Yoshimasa et al. (1987), who reported that treatment of frog erythrocytes with phorbol esters resulted in phosphorylation of the catalytic subunit of the adenylate cyclase enzyme coupled to the β-adrenergic receptor. Conversely, it is conceivable that in the LLC-PK_1 cell line, a cAMP-dependent mechanism may act to potentiate PI hydrolysis. Therefore, blockade of vasopressin-stimulated cAMP production by V_2 antagonists may lead to a diminution of vasopressin-induced phosphatidyl inositol hydrolysis. The role of the V_2 receptor-mediated cAMP pathway in modulating vasopressin-stimulated PI hydrolysis is unclear and remains to be clarified.

Alteration of the osmotic environment to which these cells are exposed affected the vasopressin-induced release of inositol phosphates. Increasing the osmolality of the incubation medium above 600 mosmol/kg H_20 resulted in a significant reduction in the amount of inositol phosphates released. It is doubtful that the hypertonicity adversely affected the viability of the cells. On the contrary, Roy and Ausiello (1981b) have shown that LLC-PK_1 cells display an increased vasopressin receptor density and an enhanced vasopressin-stimulated production of cAMP when incubated under hypertonic conditions. The opposite effects of hypertonic NaCl on vasopressin-stimulated PI hydrolysis and cAMP formation only serves to underscore the apparent interaction between these two second messenger systems in this cell system and may represent some form of negative feedback.

The physiological significance of vasopressin-induced PI hydrolysis in LLC-PK_1 cells and its regulation by osmolality is uncertain at the present time. These epithelial cells display transport characteristics not only of the distal nephron (H_20 reabsorption), but also of the proximal tubule (glucose, amino acid transport) and the medullary thick ascending limb (sodium transport) (Horster and Stopp, 1986). In this regard, it should be noted that an increase in peritubular osmolality has been shown to modulate the effect of vasopressin on NaCl transport and cAMP formation in epithelial cells of the medullary thick ascending limb of Henle (Hebert et al., 1981b; Torikai and Imai, 1984). While caution should be exercised when making comparisons between in vitro and in vivo situations, it is tempting to speculate that vasopressin-stimulated PI hydrolysis may be involved in NaCl transport or cell volume regulation of renal epithelial cells and these hormonal effects may be modulated by peritubular osmolality.

In summary, this study represents the first demonstration that vasopressin stimulates phosphatidyl inositol hydrolysis in a time- and dose-dependent manner. Furthermore, the response appears to be hormone-specific, in that none of the other agents tested caused a release of inositol phosphates. The vasopressin-mediated response could be modulated by altering the osmotic conditions of the incubation medium; a situation expected to be encountered in the corticomedullary gradient of peritubular osmolality in the kidney. Because the vasopressin-stimulated adenylate cyclase system is well-documented in this cell line, the LLC-PK_1 system represents an

experimental model to study the interaction of the two second messenger systems in epithelial cells of renal origin.

CHAPTER 7
SUMMARY

Arginine^{8}-vasopressin, a neurohypophyseal hormone, is synthesized as a prohormone in the supraoptic and paraventricular nuclei of the hypothalamus, transported and modified axonally and stored in nerve terminals within the neural lobe of the posterior pituitary gland. An extrahypothalamic distribution of vasopressinergic fibers also innvervates multiple regions of the central nervous system, with an especially dense field of terminals found in the brainstem. The peripheral and central vasopressin systems act, by vastly different mechanisms, to maintain fluid balance and circulatory homeostasis. Centrally, vasopressin can affect circulatory control by modulating the activity of the baroreceptor reflex and more directly, by affecting sympathetic and parasympathetic outflow. In the periphery vasopressin is a pressor substance, acting on resistance vessels and causing vasoconstriction. More importantly, vasopressin represents the principal hormone of fluid balance, acting at discrete tubular sites in the kidney to promote the reabsorption of water and electrolytes, principally sodium. Therefore, it is not surprizing that the regulation of vasopressin synthesis and release should be governed by both osmotic stimuli, mediated by centrally-located osmosensitive cells and non-osmotic stimuli, sensed by volume-sensitive mechanoreceptors in the heart and peripheral vessels. Alterations in the synthesis or release of the hormone can have

profound physiological consequences. These range from diabetes insipidus, a condition characterized by an inability to synthesize vasopressin and manifested clinically by polydipsia and polyuria, to the syndrome of inappropriate ADH secretion wherein circulating levels of vasopressin are dramatically elevated, promoting fluid retention and often resulting in hyponatremia.

Conversely, the biological activity of vasopressin can be affected by modifying the sensitivity of the target tissue to the hormone. AVP initiates a biological response, be it central, renal, vascular or otherwise, by interacting with plasma membrane-bound receptors located on the cells of the target tissue. These receptors are integral proteins which transduce the principal (hormone) signal into a cellular response by activation of "second messenger" systems. Currently, vasopressin receptors have been classified into two subtypes (Michell et al., 1979). The V_1 receptor is coupled to the activation of phosopholipase C, resulting in the hydrolysis of phosphatidyl inositol and the generation of inositol phosphates (IP) and diacylglycerol (DG). DG is an endogenous activator of protein kinase C, an enzyme which causes the phosphorylation of numerous cells proteins. IP acts to cause the release of calcium from intracellular stores, which in turn, can affect the activity of calcium-dependent enzymes (Berridge, 1987). V_1 receptors have been characterized in smooth muscle tissue where they mediate the vasoconstrictive actions of the hormone. In addition V_1 sites have also been reported on glomerular mesangial cells and in gonadal, liver and adrenal tissue (Chapter 1). In addition, it now apprears that V_1-type receptors are also present in epithelial cells of renal origin

(Chapter 6). V_2 receptors, thought to located exclusively on renal tubular epithelial cells, are coupled to adenylate cyclase and the formation of cyclic AMP (Morel et al., 1987). These receptors are responsible for transducing the antidiuretic action of the hormone. By means of cAMP-dependent protein kinases, phosphorylation of integral proteins in the apical membrane of a discrete population of epithelial cells leads to the formation of aqueous channels (Gluck and Al-Awqati, 1980) and an enhanced apical permeability to tubular fluid. Vasopressin-stimulated, cyclic AMP-dependent activation of Na-K-ATPase, the active transporter of sodium may account for the ability of certain epithelial tissues to promote reabsorption of this electrolyte (Tomita, et al., 1987). Clearly, modification of these receptor-mediated processes will have as dramatic an impact on physiological function as did the alteration of the hormone itself. Pathophysiological conditions associated with receptor defects are most dramatically depicted in cases of hypercholesterolemia and non-insulin-dependent diabetes mellitus, where low density lipoprotein receptor and insulin receptor numbers are diminished, thereby reducing the sensitivity of the target tissues to the respective hormones. By analogy, if the sensitivity of renal tubular target tissues to vasopressin was altered, this could have profound effects on the transepithelial transport of fluid and electrolytes, with the potential for influencing fluid homeostasis.

In this dissertation, the effect of alterations in fluid balance on vasopressin receptors was studied. Vasopressin receptors were characterized on basolateral membranes prepared from rat kidney tubular epithelia. Basolateral membranes were isolated by differential

and density gradient centrifugation techniques. Activity of Na-K-ATPase, a marker enzyme for these membranes, was increased ten-fold. Binding of a tritiated radioligand was rapid, reversible, saturable and specific. The binding site displayed a high affinity (~1nM) and fit a pharmacological profile consistent for a V_2-type vasopressin receptor. Furthermore, AVP-stimulated cAMP formation was demonstrated using this membrane preparation.

Vasopressin receptors undergo regulation, in vivo and in vitro. Homologous downregulation has been demonstrated previously, but only under the influence of pharmacological doses of AVP (Rajerison et al, 1977; Lester et al., 1985; Eggena and Ma, 1986). What effect, if any, do physiological changes in circulating levels of vasopressin have on renal tubular vasopressin receptors? The present study showed that V_2 vasopressin receptor number is reduced in basolateral membranes prepared from water-deprived rats. This has important ramifications with respect to fluid homeostasis, especially in an animal already compromised by dehydration. One plausible explanation in this case is that the downregulation may reflect a diminution of a vasopressin receptor "reserve" (Ariens et al., 1960). "Spare" receptors have been noted in other adenylate cyclase-linked tissues as well (Flores et al., 1974). Alternatively, a fraction of the total number of receptors present in any given tissue may represent "silent" receptors (Maack et al., 1987). These binding sites are not coupled to a biological effector system and therefore, elicit no response. However, they may serve as "peripheral clearance sites", acting as a buffer system to modulate plasma levels of the hormone. Thus, downregulation of the inert hormone-receptor complex may

represent one avenue for buffering the system against high circulating levels of the hormone. Evidence is accumulating that receptor reserve and signal transduction relationships are impacted by changes in physiological state (Kinter et al., 1988). The physiological significance of "silent" and "spare" receptors and their regulation remains to be explored.

Heterologous regulation of vasopressin receptors implies that other hormones or factors can regulate AVP receptor expression. Roy et al. (1980) described the regulation of vasopressin receptors by insulin, in an in vitro preparation. In the present study, the expression of vasopressin receptors and vasopressin-stimulated adenylate cyclase activity was dramatically increased in rats treated with a synthetic steroid hormone, desoxycorticosterone acetate (DOCA) + 1% NaCl. The upregulation occurred despite an elevated plasma concentration of vasopressin. This suggests that the increased density of AVP receptors is due to an increased transcriptional rate of vasopressin receptor protein synthesis, induced by the steroid hormone. Desoxycorticosterone acetate (DOCA) bas previously been shown to affect the expression of other basolateral membrane proteins, including Na-K-ATPase (Garg et al., 1981). Reif et al. (1986) have demonstrated that vasopressin-stimulated sodium reabsorption is augmented in the presence of DOCA. Taken together with the findings of Pettinger et al. (1986) that vasopressin-stimulated cyclic AMP formation was enhanced in microdissected cortical collecting tubules of DOCA-treated rats, the above studies suggest that the tubular effects of vasopressin play a role in the etiology of mineralocorticoid-dependent hypertension. The present

findings indicate that transporting-epithelial tissue in the kidney display an increased sensitivity toward vasopressin, as indicated by the enhanced formation of AVP-stimulated cyclic AMP. The increased fluid and sodium-retaining properties of the hormone lend themselves to the development of hypertension, exclusive of its vasopressor activity. The role of the kidneys in the pathophysiology of hypertension is supported by Guyton et al. (1972) and the observations of Manning et al. (1979) and Cowley et al. (1984).

Regulation of vasopressin receptors in vitro has been reported by Roy et al. (1980) and Lester et al. (1985). The lack of a demonstable effect of DOCA in LLC-PK_1 cells in the present study may reflect an absence of receptors for mineralocorticoids in this cell line. This is supported by the findings of Roy et al. (1980) who were unable to modulate receptor expression by aldosterone in LLC-PK_{1L} cells, a subline of the parent LLC-PK_1 strain. This contrasts with the ability of mineralocorticoids to exert effects on physiological processes in A_6 and MDCK epithelial cell lines (Meier and Insel, 1985). Methodological considerations should not be overlooked however, when interpreting the results of DOCA-treated LLC-PK_1 cells.

Perhaps the most exciting finding in this series of studies is that vasopressin is coupled to two distinct receptor subtypes in LLC-PK_1 cells. This exciting discovery would redefine the long-held dogma that V1 receptors are present on a variety of tissues, but that transport-epithelia possess only V_2 receptors, linked to adenylate cyclase. The present study demonstrated that indeed, vasopressin-stimulated cyclic AMP formation does occur in these cells. By

loading ^{3}H-inositol into the cell membrane, it was shown that vasopressin was also effective in stimulating the hydrolysis of phosphatidyl inositol which had incorporated the radioligand. This resulted in the release of ^{3}H-inositol phosphates which, according to Berridge (1987), act to mobilize intracellular stores of calcium. This finding complements the study of Tang and Weinberg (1986) who measured vasopressin-stimulated calcium fluxes in the same cell line. In addition, release of inositol phosphates in the present study was modulated by altering the ionic milieu of the incubation buffer. This effect mimicked the changing osmotic composition found in the corticomedullary gradient of interstitial fluid in the kidney. Whereas IP released was diminished with increasing osmotic concentration, Roy and Ausiello (1981b) reported that vasopressin-stimulated cyclic AMP production increased with increasing osmotic concentration. This underscores the potential interaction between the two transmembrane signaling systems.

Therefore, according to the classification scheme of Michell et al. (1979), it appears that LLC-PK$_1$ cells possess not only V_2-type receptors linked to adenylate cyclase but that V_1-type receptors coupled to PI hydrolysis are also present on the cell surface. The present finding and those of Roy and Ausiello (1981b) suggest that vasopressin is capable of regulating multiple effector systems. Conversely, these signal transduction systems appear to be regulated differentially by the ionic milieu to which they are exposed. This may allow for one action of vasopressin to prevail in a particular segment of the nephron. Only very recently reports have surfaced in the literature describing the expression of multiple vasopressin

receptor subtypes in renal epithelial tissue (Star et al., 1988; Burnatowska-Hledin and Spielman, 1988). Even more extraordinary, a single muscarinic receptor subtype (M_2) has been shown to be coupled to both adenylate cyclase and phosphoinositide hydrolysis (Ashkenazi et al., 1987).

These studies were intended to further our understanding of vasopressin receptors and receptor-mediated signal transduction processes in renal epithelial tissue. The series of experiments demonstrate:

1) The characterization of vasopressin binding sites in a Na^+-K^+-ATPase-enriched preparation of renal tubular epithelial basolateral membranes prepared by density-gradient centrifugation;

2) Dehydration-induced elevation of circulating levels of vasopressin down-regulates vasopressin receptors;

3) Excess exposure to mineralocorticoids up-regulates vasopressin receptors and increases the tubular sensitivity of vasopressin-stimulated adenylate cyclase. Enhanced sensitivity to the fluid-retaining properties of the hormone provides a cellular and systemic basis for the role of vasopressin in the pathogenesis of DOCA-salt hypertenion;

4) Vasopressin receptors are coupled to the formation of cAMP, and also to phosphatidyl inositol turnover in LLC-PK_1 cells. The two transduction processes are modulated differentially by the osmotic strength of the incubation medium. The coupling of vasopressin receptors to multiple effector systems redefines our concept of signal transduction in renal tubular epithelia.

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BIOGRAPHICAL SKETCH

Martin Steiner was born March 3, 1952, in New York City, where he lived for a period of one year. He moved with his family to Chicago, where he remained for the next 17 years. He attended the University of Iowa where he received a B.S. degree in zoology in 1974. After five years as a research assistant in the Departments of Pharmacology and Pyschiatry at The University of Iowa, he began his graduate studies, receiving a M.S. degree in zoology from Clemson University in 1982. He proceeded to the University of Florida and entered the graduate program in physiology.

Under the supervision of Dr. M. Ian Phillips, Martin Steiner was awarded a Ph.D. in April, 1988. There is no truth to the rumor that he will pursue other courses of study, thereby ending his quest to become the longest living "professional student." Therefore, he expects to a) get married, b) get a job, and c) live happily ever after.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

M. Ian Phillips, D.Sc., Chairman
Professor of Physiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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